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Studies on the Encystment of Azotobacter Vinelandii.

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Studies on the Encystment of *Azotobacter vinelandii*

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ABSTRACT

Cysts of Azotobacter vinelandii ATCC 12837 have been produced in batch culture utilizing Burk's nitrogen-free medium with 1% glucose as the source of carbon in the presence of 0.6% powdered $CaCO_3$. Mature cysts produced by this method possess properties similar to those of cysts induced by replacement of the glucose-containing medium with 0.1% β-hydroxybutyrate-containing medium. When 0.1% β-hydroxybutyrate was added to the glucose-containing medium at 18 h of growth, abortive encystment occurred. These cultures became extremely viscous and free exine-like structures were evident throughout the medium.

The ultrastructure of vegetative cells and cysts of Azotobacter was examined utilizing various techniques of scanning and transmission electron microscopy. Intact colonies were prepared for scanning electron microscopy by critical point drying. This method of specimen preparation enabled examination of cells in situ without introduction of artifacts associated with surface tension during drying. These studies confirmed the presence of vesicles originating from the cell wall of encysting cells during morphogenesis. Samples were removed from batch cultures at 12 h intervals throughout the 6 day encystment period. These samples were prepared for thin sectioning by a double-fixation procedure accomplished in the presence of ruthenium red. Thin sections examined by transmission electron microscopy revealed the origin of the vesicles to be the L-layer of

the cell wall. These vesicles were produced from 12 h to 36 h of growth. The vesicles, containing lipopolysaccharide (LPS) from the cell wall, accumulated in the capsule and were subsequently incorporated into cyst coat structures.

Cysts were fractionated into exine and intine components by treatment with 3 mM EDTA. The exine was isolated as a particulate fraction by differential followed by density gradient centrifugation. Intine, which was solubilized by EDTA, could be recovered by ethanol precipitation.

Chemical extraction procedures were utilized for selective dissection of the cyst coats. EDTA effected the release of a polysaccharide and a LPS component. Phenol extracted only LPS.

The LPS was quantitated by the carbocyanin dye assay. Supernatants from nonencysting cultures contained large amounts of free LPS. LPS was present in encysting cultures until 12 h of growth after which time, the amount of LPS rapidly declined. This appearance of LPS is thought to result from evagination of vesicles during encystment. Disappearance of LPS results as calcium ions coordinate the LPS into a structural component of cyst coats.

The chemical analysis of cyst coat fractions has enabled a better understanding of encystment. Capsular polysaccharide of Azotobacter contained glucose and a hexuronic acid. Exine composition reflected capsule composition with the additional LPS-specific components rhamnose, ribose, and glucosamine. Intine contained both polysaccharide and LPS components, including galactose, xylose, ribose, rhamnose, and galactosamine. The purified LPS was hydrolyzed

for isolation of lipid A. LPS composition included ribose, rhamnose, heptose and galactosamine. The presence of ethanolamine and a 2-keto-3-deoxy sugar is suspected. The lipid A component contained derivatives of C-12 and C-14 fatty acids.

A modification of the present theory of encystment is proposed. The first morphological event of encystment is the evagination of vesicles as early as 12 h of growth. This LPS accumulates in the culture medium. When sufficient calcium is present, the capsule is altered in such a manner that polysaccharide and LPS are coordinated into the differentiated structures of cyst coats. Flagella are entrapped in this process, resulting in the small amount of protein present in cyst coat fractions.

INTRODUCTION

The formation of bacterial resting forms provides microbiologists with elegant model systems for elucidation of the regulation of cellular differentiation and morphogenesis. The resting forms most frequently studied include the endospores produced by Clostridium and Bacillus, the microcyst produced by Myxococcus, and the cyst produced by Azotobacter.

While endospores and microcysts have been the subject of long-standing research, cysts of Azotobacter remain poorly characterized chemically, physiologically and genetically. Only the ultrastructure and morphogenesis of these forms have been extensively examined.

Development of liquid culture methods for the production of cysts has removed many of the barriers to chemical and physiological characterization. However, discrepancies in cyst production methods have led to confusion concerning the ability of cysts to form in a glucose-containing medium. Furthermore, morphological and ultrastructural investigations conflict because of the variety of preparative methods utilized in preservation of samples for electron microscopy.

This research was undertaken to examine definitively the ultrastructure of vegetative cells and cysts of Azotobacter vinelandii with special emphasis on the morphogenesis leading to the cyst state. Information about the structure of the cyst coat

obtained from this study served as a theoretical basis for the selection of fractionation methods for extraction and isolation of the various components of the cyst coat. The chemical analysis of these fractions was undertaken in an attempt to determine the origin of components and the relationship of structure and function in the encystment of Azotobacter.

REVIEW OF LITERATURE

Azotobacter vinelandii has been the subject of much research directed toward the elucidation of the mechanisms of the cellular differentiation occurring during encystment. This problem has been investigated by divergent approaches employing developmental, morphological, and physiological methods.

Cysts of Azotobacter

Jones (1920) first recognized and reported a thick-walled resting form in 14-day cultures of Azotobacter. In 1935, Batchinskaya reported a double-layered capsule surrounding aging cells of Azotobacter. Microscopic observations led him to suggest that the innermost layer was composed of a gel-like material, encapsulated by a denser outer layer.

The first studies of encystment consisted of attempts to induce cyst formation by incorporation of various substrates into the culture medium. Winogradsky (1938) found that vegetative cells could be induced to encyst rapidly and completely by incorporation of n-butanol as a sole source of carbon into the culture medium. He described the cytology of these cysts by examining violamine-stained preparations, observing a darkly-staining inner portion corresponding to the modified vegetative cell, a yellow staining inner ring, and a darkly staining outer ring. The inner and outer layers of the cyst coat were termed intine and exine, respectively.

The organism remained little more than a laboratory curiosity for the next several years, during which time several investigators reported the occurrence of various bizarre morphological variants including filaments, buds, arthrospores, endospores, exospores, microcysts, and gonidia (Löhnis and Smith, 1923; Eisenstark, et al., 1950; Bisset and Hale, 1953). It is currently believed that most of this pleomorphism arose from observations of contaminated cultures or by incorporation of combined nitrogen sources into the culture medium (Jensen, 1954).

The next serious attempt to study encystment was undertaken by Socolofsky and Wyss (1961), who demonstrated the structure proposed by Winogradsky (1938) by examining cysts in thin sections prepared for electron microscopy. These investigators found that ethylenediamine tetraacetic acid (EDTA) effected rupture of the exine with subsequent release of the central body in a nonviable state. The efficiency of release could be followed by the associated decrease in optical density of the cyst suspensions. Since vegetative cells were rather insensitive to EDTA exposure, lytic susceptibility became a criterion of cyst maturity. This action of EDTA led to the hypothesis that divalent cations were active in coordination of cyst coat structures. Socolofsky and Wyss (1962) found that cysts were extremely resistant to desiccation, providing another criterion of encystment and an explanation for the occurrence of the cyst state in nature.

Wyss, et al. (1961) examined the morphogenesis of cysts, induced by the method of Winogradsky (1938), and prepared for electron microscopy by $KMnO_4$ fixation. As encystment proceeded

rod-shaped vegetative cells rounded up, accumulated multiple inclusion granules presumed to be poly-β-hydroxybutyric acid (PHB), and proceeded to acquire a multi-layered coat.

Tchan, et al. (1962) obtained similar results by studying cultures fixed with 1% OsO_4 (Kellenberger, et al., 1958). They reported a discontinuous trilaminar structure occurring in the intine layer of the cyst coat.

Stevenson and Socolofsky (1966) noted a direct correlation between the amount of PHB accumulation and the percentage of cysts formed. They suggested that PHB accumulation occurred concomitantly with carbon assimilation since exogenous carbon was utilized more rapidly than the cells could fix atmospheric nitrogen. This unbalanced growth condition was necessary for encystment since PHB could be mobilized for incorporation into cyst coat components after exogenous carbon assimilation and nitrogen fixation had ceased. Thus, these investigators felt that PHB provided the carbon and energy sources for encystment.

Senior and Dawes (1971) suggested that PHB accumulation served as a mechanism by which a readily available reserve of carbon, reducing power, and energy also served in a regulatory capacity by channeling NADPH and NADH, involved in its biosynthesis, to impose limitations on growth through inhibition of glucose oxidation and citrate metabolism, thus controlling the rate of nitrogen fixation.

Cohen and Johnstone (1963) reported that when Azotobacter vinelandii was grown in liquid culture, acid polysaccharides were excreted into the medium lowering the pH to 5.5 after five days of growth. Cells were extremely sensitive to acid pH and no encystment

occurred under those conditions. Stevenson and Socolofsky (1972) modified Burk's nitrogen-free salts solution (Wilson and Knight, 1952) by substituting $CaCl_2$ for $CaSO_4$ and by the addition of 0.6% powdered $CaCO_3$, to maintain an alkaline pH and to provide an excess of calcium to encourage encystment. Cells were quantitatively converted into cysts in four days, when 1% glucose served as the carbon source. Criteria for encystment included lytic susceptibility, desiccation resistance, and optical refractility adjudged by phase contrast microscopy. Unbuffered cultures or cultures periodically neutralized with KOH accumulated considerable amounts of acid polysaccharide noted by the low pH and extreme viscosity of culture supernatants. Using their newly developed liquid culture method, Stevenson and Socolofsky (1973) confirmed the relationship between PHB accumulation and percentage of encystment by following the distribution of ^{14}C-labeled acetate in cysts. Greater than 90% of the label was found in PHB when cultures were sampled in the precystic stage of development. When $CaCO_3$ was present in the culture medium, 80% of the label remained identified with the cysts while 20% was lost by CO_2 evolution. Of the 80% contained in cysts, fractionation with EDTA resulted in 65% of the label appearing in the central body and exine (particulate fraction) and 35% of the label appeared in what was presumed to be intine (soluble fraction). When no $CaCO_3$ was employed, label distribution was 20% in CO_2, 45% in the PHB-cell fraction and 35% in the polysaccharides of the culture supernatant.

Lin and Sadoff (1968) contended that cells of Azotobacter vinelandii could not be induced to encyst in a glucose-containing

medium. They examined several compounds metabolically related to n-butanol, including butyraldehyde, butyrate, crotonate, and β-hydroxybutyrate (BHB). Only crotonate and BHB were successful encystment inducers. These results led to the two-step replacement culture method where log phase vegetative cells were produced in Burk's nitrogen-free medium with 1% glucose as the carbon source. At 18 h of growth, the cells were pelleted, resuspended in Burk's salts solution, washed, and resuspended in Burk's medium with 0.1% BHB as the carbon source. Encystment was complete four days after replacement.

When cysts were produced by BHB induction, cultural conditions paralleled those described by Stevenson and Socolofsky (1973). Cultural viscosity remained low and encystment reached 93% in five days based upon direct counts of optically refractive bodies by phase contrast microscopy.

Attempts were made by Lin and Sadoff (1968) to induce cysts by supplementing the glucose-containing medium with 0.1% BHB, but those cultures became extremely viscous and few cells encysted. These results were similar to those reported by Stevenson and Socolofsky (1973) where cultures were neutralized periodically with KOH. Lin and Sadoff (1968) concluded that glucose antagonized the effect of BHB to promote encystment. They suggested that the increased viscosity of culture supernatants was the result of non-crosslinked peptidoglycan excreted from the cell.

Physiology of encystment

Development of liquid culture methods for production of cysts enabled detailed investigation of the physiological alterations occurring during encystment.

When cells of Azotobacter vinelandii were grown by the replacement culture method, they underwent a final round of cell division without apparent synthesis of deoxyribonucleic acid (DNA), thereby reducing the number of nucleoids from two to one per cell (Sadoff, et al., 1971). According to these investigators, BHB dehydrogenase was derepressed by addition of the inducer but this single event was not responsible for induction of encystment. A complete cessation of nitrogen fixation occurred three hours after replacement.

Hitchins and Sadoff (1973) extended these investigations of cyst induction which employed the replacement method by characterizing the sequence of biochemical events occurring in the first 36 h of encystment. Upon initiation of encystment, nitrogen fixation and glucose-6-phosphate dehydrogenase activity decreased, followed by a biphasic increase in the activity of BHB dehydrogenase, isocitrate dehydrogenase, isocitrate lyase, and malate synthetase at 3 h and at 21 h. Fructose-1,6-diphosphate aldolase activity reached a maximum at 6 h of encystment and then gradually decreased. Fructose-1,6-diphosphatase activity reached a maximum at 9 h and again at 27 h of encystment. DNA synthesis occurred up to the last cell division at 4 to 6 h of encystment while ribonucleic acid (RNA) synthesis continued until 12 h of encystment. Incorporation of ^{14}C-labeled leucine indicated protein synthesis occurred throughout encystment.

These workers concluded that polymers required for cyst coat formation must be synthesized during encystment from the lipoidal inducers via the glyoxylate shunt and gluconeogenesis. Since no nitrogen fixation occurs after initiation of encystment, macromolecular synthesis is dependent upon turnover of RNA and protein. These results parallel the biochemical events occurring in microcyst formation in Myxococcus xanthus (Sudo and Dworkin, 1973) and in sporulation of the Bacillaceae (Vinter, 1969). Sadoff (1973) has reviewed the comparative aspects of the mechanisms of cellular differentiation in Azotobacter, Myxococcus, and Bacillus.

Ultrastructure of Azotobacter

Development of the liquid culture techniques enables investigators to examine the ultrastructure of Azotobacter over a wide range of conditions including cell-free extracts, cyst components, and morphogenesis under various cultural conditions. The variety of preparative techniques utilized for electron microscopy has yielded much useful information concerning the ultrastructure of Azotobacter.

Vegetative cells

Negative stains and carbon replicas of vegetative cells reveal peritrichous flagellation. Examination of thin sections has revealed the vegetative cell to be oval to rod-shaped, possessing the multi-layered cell wall typical of gram negative organisms (Hitchins and Sadoff, 1970). When cells were fixed with 1% OsO_4, the outer layer of the cell wall stained darkly and demonstrated the trilaminar structure typical of unit membranes. A second

trilaminar layer occurred but this layer did not have the high affinity for the fixative seen in the outer layer. The third trilaminar structure consisted of the cytoplasmic membrane. The investigators observed that the cytoplasmic membrane possessed numerous invaginations forming electron transparent areas first termed peripheral bodies by Wyss, et al. (1962). Gardner (1969) used tetrazolium blue to localize cytochemically the respiratory apparatus in these areas. Oppenheim and Marcus (1970) suggested the nitrogenase complex was located in the peripheral bodies. They demonstrated that the complexity of the membrane invaginations is dependent upon the partial pressure of oxygen in the culture medium. They presented evidence indicating that increased membrane complexity resulted from high levels of dissolved oxygen while lower levels reduced the degree of membrane invagination. The deleterious effect of oxygen upon the nitrogenase complex was discussed by Stewert (1973) and by Streicher and Valentine (1973). Localization of the nitrogenase complex in the peripheral bodies was confirmed by cytochemical studies using conjugated antibody to localize specifically the ferritin component (Stasny, et al., 1973 and Stasny, et al., 1974).

The cytoplasmic areas of Azotobacter appear densely packed with ribosomes and electron transparent areas corresponding to portions of the nucleoids. Microtubules occur frequently although their composition and function remain unknown (Pope and Jurtshuk, 1967).

Capsular polysaccharide is not preserved by the usual fixation procedures; however, Pope and Wyss (1970) successfully stained Azotobacter capsular polysaccharide by fixing their specimens in

the presence of ruthenium red by the procedure of Pate and Ordal (1967). The capsule appeared as a fluffy fibrous material surrounding the cells. Cagle, et al. (1972) examined a variety of slime producing bacteria stained with ruthenium red, demonstrating the usefulness of the method for visualization of encapsulated organisms.

The copious slime produced by Azotobacter results in the occurrence of aggregates of cells surrounded by a common capsule (Vela and Cagle, 1972). The purpose of this slime seems twofold - to provide a hygroscopic milieu binding soil particles into a favorable microenvironment for survival in soils (Harris and Mitchell, 1973), and to provide carbohydrate subunits for condensation into cyst coat structures (Eklund, et al., 1966).

Morphogenesis

The first ultrastructural alteration associated with encystment is the accumulation of PHB. The rod-shaped vegetative cells round up, decrease in size and become non-motile. PHB accumulation reaches a maximum at approximately 48 h after initiation of encystment. Evaginations of the wall appear on the cell surface and pinch off to form spherical vesicles. As encystment proceeds, the fibrous layers of the intine are formed, enclosed by the dense bark-like exine (Vela, et al., 1970). These workers reported a membrane separating the exine and intine layers of the cyst coat.

Hitchins and Sadoff (1970) described the morphogenesis of cysts induced in BHB-containing medium. They suggested that the vesicles, appearing at the cell wall, migrate outward into the capsular polysaccharide surrounding the cell, and flatten out to

form the plate-like structures comprising the exine layer of the cyst coat. They also suggested that the vesicles might contain a lipopolysaccharide-lipoprotein component.

Few structural changes occur after formation of the intine and exine layers on the fourth day of encystment. The central body further decreases in size and the amount of PHB gradually declines.

Koo and Sadoff (1969) reported attempts to freeze-fracture cysts but they succeeded only in observing the exterior of the intact cysts. Cagle, et al. (1973) succeeded in fracturing cysts and were able to examine the spatial relationships of cell aggregates. They confirmed the presence of vesicles in early stages of encystment. They also presented scanning electron micrographs of cells obtained from aqueous suspensions. Both vegetative cells and cysts were severely distorted from the effects of surface tension forces during drying and little useful information could be derived from the micrographs.

Fractionation of Azotobacter cysts

The first successful isolation of the exine component of the cyst coat was accomplished by Lin and Sadoff (1969a). They treated cysts with 3 mM EDTA in 0.05 M tris (hydroxymethyl) amino-methane (Tris) buffer, effecting release of the central body and solubilization of the intine component. The exine was isolated by differential and density gradient centrifugation while intine was recovered from the EDTA solution by ethanol precipitation. The efficiency of rupture and the purity of the exine preparation was monitored by examining negative stains and carbon replicas in the

electron microscope. Many of the exine micrographs presented contained PHB granules, indicating partial lysis of the central body which would result in the presence of various cellular contaminants in the presumably pure exine and intine preparations.

Pope and Wyss (1970) also examined the outer layers of cysts ruptured with EDTA. They reported the release of a hexagonal plate-like structure which they observed in negative stains of the exine material.

Wetegrove and Wyss (1974) reported that cells increased in density as encystment proceeded. The density of vegetative cells was 1.106 g/cm^3, whereas cysts had a density of 1.152 g/cm^3. This difference was believed to be due to alterations in chemical composition, the presence of lesser amounts of bound water or increased calcium content of the cyst.

Chemical composition studies

Studies of the chemical composition of Azotobacter began with investigations on the polysaccharides of slime and capsule. Cohen and Johnstone (1964a, 1964b) isolated and purified the slime and capsule of Azotobacter agilis and Azotobacter vinelandii and found a difference in composition between species. They indicated that this difference in chemical composition provided further justification for separate species status for Azotobacter vinelandii. They reported the presence of glucose, rhamnose, galacturonic acid, and mannuronolactone in Azotobacter vinelandii. Claus (1965) reported a 2-keto-3 deoxygalactonic acid component. Dazzio (1964)

also studied the capsule of Azotobacter vinelandii and reported the presence of glucose, rhamnose, mannose and a hexuronic acid.

Gorin and Spencer (1965) reported Azotobacter vinelandii capsule contained mannuronic acid, gulurunic acid, glucose, arabinose, ribose and rhamnose.

The lipid composition of vegetative cells of Azotobacter vinelandii was reported by Marcus and Kaneshiro (1972).

Lin and Sadoff (1969b) presented studies on the chemical composition of vegetative cells and cysts of Azotobacter vinelandii. They also analyzed the exine and intine fractions of cyst coat for total carbohydrate, protein, lipid, and ash. Amino acid composition of exine indicated a predominance of glycine, glutamic acid, aspartic acid, and alanine with lesser amounts of twelve other amino acids. Intine and exine fractions were shown to differ in all components analyzed and these differences were thought to reflect unique functions in the life cycle of the cyst.

Theories of encystment

The role of calcium

Wyss, et al. (1962) proposed the first theory of cyst formation when they suggested that units of polysaccharide were complexed by divalent cations to form exine, while intine consisted of capsular polysaccharide. Eklund, et al. (1966) provided further evidence supporting this concept by showing that non-encapsulated mutants or cells treated with phage capsule depolymerase failed to encyst.

The theory that divalent cations had a role in maintaining the structural integrity of cell wall components received indirect support

from Asbell and Eagon (1966) when they proposed that calcium was essential for integrity of cell walls of pseudomonads. The mechanism appeared to involve coordination of lipopolysaccharide (LPS) to other cell wall components by ionic bonding.

The presence of an equilibrium between calcium in the environment and the amount of bound calcium in cyst coats was suggested by Smith, et al. (1969). They reported that calcium could be removed from cysts by repeated washings in distilled water or by exchange with other cations.

Goldschmidt and Wyss (1968) studied the effect of EDTA in removal of calcium from cyst coats and concluded that the efficiency of rupture depended upon the molarity of the reaction mixture. The presence of salts above a concentration of 0.15 M NaCl or phosphate prevented the chelation effect of EDTA. These authors suggest that the high salt content of cysts is a prerequisite for desiccation resistance which enables the organism to remain viable in soil for extended periods of time.

The most cogent argument supporting the role of calcium in coordinating Azotobacter cyst structures was presented by Larsen and Haug (1971). An increase in calcium concentration of the culture medium after alginate components were present, resulted in a change in composition of the alginate which occurred even if the cells were removed by centrifugation. This observation suggested the culture medium contained an enzyme capable of epimerizing D-mannuronic acid residues to L-guluronic acid residues in the presence of calcium. The significance of this occurrence in cyst coat formation remains to be elucidated.

Another approach to the role of calcium in encystment relates to the specific localization of calcium ions by X-ray probe microanalysis. Scherrer and Gerhardt (1972) attempted the localization of calcium in Bacillus spores but found that calcium was uniformly distributed throughout the cortex.

The role of lipopolysaccharide

The remaining theory of encystment was proposed by Hitchins and Sadoff (1970) when they suggested that the vesicles of cell wall origin were coordinated to form a significant part of cyst coat structures. Because of the trilaminar structure of the vesicles, they suggested that exine contained a lipopolysaccharide (LPS) component.

The LPS from cell walls of Azotobacter vinelandii was physico-chemically characterized by Olins and Warner (1967). They reported that the LPS consisted of two polydisperse components and that treatment with EDTA resulted in dissociation to species of lower molecular weight. The LPS was found to contain glucose, ribose, rhamnose, hexosamine and a 2-keto-3-deoxy sugar.

Leive (1965) reported that treatment of Escherichia coli with EDTA resulted in release of 50% of the LPS from the cell wall. Thus, if LPS is a component of exine, rupture could result from removal of structural LPS from cyst coats as well as chelation of calcium ions.

Knox, et al. (1966) reported that lysine-limited cultures of E. coli excreted free LPS into the culture medium resulting in an increase in viscosity not unlike that occurring when cultures of Azotobacter were neutralized with KOH or cultured by the supplemental

method of Lin and Sadoff (1968). Lysine-limited growth apparently produces effects similar to those encountered in unbalanced growth of Azotobacter resulting from the lag in nitrogen fixation compared to carbon assimilation.

Studies of LPS have been greatly facilitated by the development of a colorimetric assay by Janda and Work (1971). These investigators modified the carbocyanin dye assay method of Edstrom (1969) for quantitative determination of LPS either in purified form or in culture supernatants. The further modification of the assay by Zey and Jackson (1973) resulted in a more sensitive and reliable method for quantitation of LPS.

MATERIALS AND METHODS

Culture methods

Organism and culture media

The bacterium Azotobacter vinelandii, ATCC 12837, was the subject of this research. Stock cultures were maintained in screw-capped tubes upon Burk's nitrogen-free medium with 1% mannitol as the carbon source. The composition of the medium is presented in Table 1 (Stevenson, 1967). Other carbon sources included n-butanol, glucose and BHB.

Inocula and incubation

Cultures to be used as inocula were produced by streaking cells from a stock culture onto the surface of Burk's nitrogen-free medium with 1% glucose as the carbon source and incubating the plates at 32 C in a cabinet incubator for 24 h. Vegetative cells from these plates were then inoculated into 50 ml aliquots of Burk's nitrogen-free liquid medium with 1% glucose as the carbon source and incubated at 32 C in a rotary shaker at 180 rpm for 18 h at which time the cells were in the late lag phase of growth. Inocula for all experimental cultures were produced in this manner.

Cyst production

Cysts were routinely produced on the surface of Burk's nitrogen-free liquid medium with 0.2% n-butanol as the carbon source or by addition of 0.6% powdered calcium carbonate to the liquid medium with 1% glucose as the carbon source (Stevenson, 1967).

Table 1. Composition of a modified Burk's nitrogen-free salts solution.

Component	Grams per liter of distilled water
KH_2PO_4	0.2
K_2HPO_4	0.8
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2 \cdot 2H_2O$	0.085
$FeSO_4 \cdot 7H_2O$	0.005
$Na_2MoO_4 \cdot 2H\ O$	0.0003

Two additional culture methods were utilized in attempts to confirm the results of Lin and Sadoff (1968). Cysts were induced by a "replacement" method whereby 18 h vegetative cells grown in liquid culture with 1% glucose as the carbon source were centrifuged, washed once in Burk's buffer, and resuspended in Burk's medium with 0.1% β-hydroxybutyrate as the carbon source. Encystment was complete on the fourth day after replacement. The second method consisted of supplementing the glucose-containing medium by the addition of 0.1% BHB, resulting in abortive encystment.

Chemicals

Glucostat reagent was obtained from Worthington Biochemical Corporation, BHB from Sigma Chemical Company, and orcinol from Matheson, Coleman and Bell. The orcinol was recrystallized before use according to the procedure of Schneider (1957). DNA (sperm) was provided by Cal Biochemicals and RNA was purchased from General Biochemicals. The carbocyanin dye, 1-ethyl-2- 3-(1-ethylnaphtho [1,2d]-thiazolin-2-ylidene)-2-methyl-propenyl naphtho[1,2d] thiazolium bromide was purchased from Eastman Kodak Company. All other chemicals were reagent grade obtained from commercial sources.

General procedures

Measurement and control of pH

The pH of culture media, buffers, and reagents was measured with a Corning electrode in conjunction with a Beckman Zeromatic pH meter. The pH of tris buffer was adjusted with Beckman dual probe electrodes. Control of pH in cultures was accomplished by

adding measured amounts of 1 M KOH, whereas the pH of reagents was adjusted using appropriate ionic species.

Optical density measurements

The optical density of cultures was determined at 600 nm with a Bausch and Lomb Spectronic 20 colorimeter-spectrophotometer. All other spectrophotometric measurements were accomplished utilizing a Beckman DB spectrophotometer equipped with a UV power supply and a strip-chart recorder.

Viscosity measurements

The viscosity of cultural supernatants was determined utilizing a Cannon-Fenske #200 viscometer in a water bath at 32 C. Relative viscosities were reported as efflux times compared to distilled water.

Viable counts

Quantitation of viability was accomplished by the conventional smear plate technique using distilled water dilution blanks. Samples were plated in triplicate and counts were made after incubation for 2 days at 32 C.

Determination of encystment

The degree of encystment was determined by direct counts of refractile bodies in wet mounts by phase microscopy. Percentage of encystment was determined after counting 200 cells. Cultures were routinely examined in this manner at various times throughout the course of the research.

Lytic susceptibility

The susceptibility of cysts to lysis by treatment with EDTA in tris buffer was determined by the method of Socolofsky and Wyss (1961).

Morphological investigation

Scanning electron microscopy

Intact colonies were prepared for scanning electron microscopy (SEM) by streaking plates with cells and incubating the plates for various periods of time. Blocks of agar containing 2-10 colonies were cut from the plates and immediately passed through an ethanol dehydration series and infiltrated with a 1:1 preparation of amyl acetate-ethanol followed by exposure to amyl acetate. The blocks could be stored in the amyl acetate indefinitely. The blocks were placed in the chamber of a Denton CPD-1 critical point drying apparatus, flushed with liquid CO_2 for 10 min and passed through the critical point at 1065 psi by warming the chamber to 55 C. The chamber was then vented and the blocks were removed and stored in a desiccator over NaOH.

Specimens were attached to aluminum stubs with silver adhesive and coated with 200 Å of gold in a vacuum evaporator equipped with a rotary stage. The specimens were examined in a JOELCO scanning electron microscope at an accelerating potential of 25 kV. Photographs were made directly from the cathode ray tube onto Kodak Tri-X Ortho film.

Transmission electron microscopy

Negative stains

Negative stains of lipopolysaccharide material were prepared by placing 0.3 ml of the preparation at a concentration of 2 mg/ml into a tube and adding one drop of 2% phosphotungstic acid, pH 7. The mixture was allowed to stand at room temperature for 30 min after which time a

drop of the mixture was placed on a surface of dental wax and a 300 mesh carbon-coated grid was inverted on the drop for 5 min. The grid was allowed to air dry at room temperature and was examined by transmission electron microscopy (TEM) in a RCA EMU-3G electron microscope with an accelerating voltage of 50 kV.

Thin sections

Specimens were fixed in 3.6% glutaraldehyde for 1 h in an ice bath and post-fixed with 4% OsO_4 for 3 h at room temperature. Cacodylate buffer (pH 7.4, 0.15 M) was used in the preparation of the reagents and in all subsequent procedures. Both fixation procedures were accomplished in the presence of ruthenium red (1.5 mg/ml). Samples were dehydrated in ethanol and embedded in Spurr resin. Sections were cut using an LKB Ultratome with a diamond knife and post stained with uranyl acetate followed by lead citrate. Examination of the samples was accomplished in the electron microscope at an accelerating potential of 50 kV.

Fractionation methods

Preparation of cyst coat components

Exine

Liquid-grown cysts were washed in Burk's buffer and resuspended in 3 mM EDTA in 0.05 M Tris, pH 8.0, to an optical density of 0.8. The suspension was shaken at 32 C for 15 min and centrifuged by the method of Lin and Sadoff (1969a).

The final pellet containing crude exine was resuspended in 5 ml of distilled water and layered on 45% sucrose. The discontinuous gradients were centrifuged at 12,000 rpm for 3 h. The top layer was

removed and washed three times with distilled water to remove the sucrose. The purified exine was lyophilized and stored in a desiccator over NaOH.

Intine

The EDTA-soluble material, derived from the intine portion of the cyst coat by the method of Lin and Sadoff (1969), was concentrated five-fold by flash evaporation and precipitated with 95% ethanol acidified with 0.1% HCl. The preparation was allowed to stand overnight at 4 C to insure fullest recovery. The precipitate was collected by centrifugation, dissolved in distilled water, reprecipitated, and collected by filtration. The precipitate was dried and stored in a vacuum desiccator over flake NaOH.

Capsule and slime extractions

Cultural slime and capsule fractions were prepared from 18 h vegetative cells and 4 day mature cysts by the methods of Cohen and Johnstone (1964b). The individual samples were hydrolyzed with 0.1 N HCl in sealed evacuated ampules at a concentration of 5 mg/ml by placing the ampules in an oven at 100 C for 48 h. The HCl was removed by evaporation in vacuum and the samples were silylized for gas chromatographic analysis of the carbohydrates.

LPS extraction

Aqueous phenol method

LPS was extracted by the method of Westphal, et al. (1952) from intact cysts. The crude LPS was dialyzed 48 h against 6 changes of 10 mM $MgCl_2$ and collected by centrifugation at 105,000 x g for 3 h. The clear jelly-like pellet was resuspended in distilled water

and the 260/280 nm ratio was determined. The concentrated LPS was treated with RNAse, dialyzed, and lyophilized. Purified LPS was stored in a desiccator over NaOH.

EDTA method

LPS was extracted from cysts by the method of Leive, et al. (1968). This LPS fraction was stored over NaOH. The EDTA-extracted cysts were then subjected to the phenol extraction procedure to remove any remaining LPS.

Chloroform method

Culture supernatants were extracted with chloroform by the method of Knox, et al. (1966). These extracts were tested by the carbocyanin dye assay for the presence of LPS.

Lipid A isolation

Lipid A was isolated according to the method of Hartley, et al. (1974). Glacial acetic acid was added to 30 mg of purified LPS in aqueous solution to a final concentration of 1% and the solution was heated at 100 C for 90 min. The flocculant precipitate formed consisted of crude lipid A which was recovered by centrifugation. The crude lipid A was dissolved in chloroform and reprecipitated with acetone. This purified lipid A fraction was refluxed in a mixture of 4.2 ml of methanol and 0.8 ml of concentrated HCl for 8 h. The mixture was cooled and extracted with petroleum ether to solubilize the fatty acid components. The petroleum ether extract contained fatty acid methyl esters and was designated fraction I. The ether-insoluble material contained deacylated lipid A and was designated fraction II.

Analytical methods

Hydrolytic methods

Hydrolysis of the samples under investigation was accomplished by placing 4 mg samples in ampules with 2 ml of various concentrations of hydrochloric acid. The ampules were then sealed under vacuum and hydrolyzed according to the schedule presented in Table 2. The ampules were then opened and evaporated to dryness under vacuum over flakes of NaOH in the presence of sulfuric acid. Samples were redissolved in 2 ml of distilled water and reevaporated to dryness two times after which they were stored in a desiccator under vacuum.

Paper chromatography

Dried hydrolyzed samples were redissolved in 1 ml of distilled water and 10 ul was applied to sheets of Whatman #1 chromatography paper. Ascending chromatography was carried out using the following solvent systems: n-butanol, pyridine, water (6:4:3); isopropanol, pyridine, acetic acid, water (5:5:3:1); and n-butanol, acetic acid, water (50:15:35). Sugars were localized by spraying the chromatographs with ninhydrin, aniline phthlate, anisidine, phenylenediamine (Putman, 1957) or thiobarbituric acid (Warren, 1956). Additional chromatograms were developed by the silver nitrate dip method of Horrochs and Manning (1949).

Colorimetric methods

Glucostat reagent was utilized for the determination of glucose. Protein was determined by the method of Lowry, et al. (1951) or by absorbance at 280 nm. Orcinol and diphenylamine reagents were employed for the measurement of RNA and DNA, respectively

Table 2. Conditions of hydrolysis of cyst coat fractions at 100 C.

Acid	Concentration, normal	Time, hours	Reducing equivalents, mg glucose/ml
HCl	0.1	4	32
		6	28
	0.5	14	80
		16	62
	1.0	1	0
		2	60
		3	45
		4	20
	4.0	4	48
		6	66
	6.0	4	10
		8	22
		12	34
		16	47
		20	56
		24	68
H_2SO_4	1.0	8	0
	2.0	4	1
		6	4

(Schneider, 1957). Total hexose was measured by the phenol-sulfuric acid method of Dubois, et al. (Ashwell, 1966) with glucose as the standard. Hexosamine was estimated by a modification of the Elson-Morgan procedure (Davidson, 1966). Heptose and methyl pentose were determined using the Dische reactions with standards of sedoheptulose and rhamnose, respectively. 2-Keto-3-deoxy sugar determination was accomplished with the 2-thiobarbituric acid reagent in attempts to identify the 2-keto-3-deoxyoctulasonic acid (KDO) component characteristic of LPS of other organisms. No authentic KDO was available for comparison. Reducing power of hydrolysates was determined by the Nelson-Somogyi reagent with glucose as the standard.

Carbocyanin dye assay

LPS was identified qualitatively and semi-quanitatively by a modification of the carbocyanin dye method of Edstrom. The dye reagent was prepared according to the method of Zey and Jackson (1973). The dye solution was clear with a deep magenta color and was stable at 4 C for 8 h. The dye was prepared for use by adding 0.5 ml of 0.01 M ascorbic acid to a 25 ml aliquot of the reagent. This dye reagent was unstable and had to be used within one hour after addition of the ascorbic acid. For performance of the assay, a quantity of 1 ml of the sample under test was added to 0.14 ml acetate buffer and mixed well. Subsequently, 0.6 ml of the dye reagent was added and allowed to react 10 min at room temperature after which the optical density was read against a dye blank at 472 nm.

Gas chromatography

The samples to be analyzed by gas chromatography were hydrolyzed in sealed, evacuated ampules at a concentration of 5 mg/ml in 0.1 N HCl at 100 C for 48 h. The ampules were cooled, opened and transferred to 2 dram plastic-stoppered vials for evaporation of the HCl in a vacuum desiccator over flaked NaOH in the presence of H_2SO_4. After the samples had been evaporated to dryness, 2 ml of distilled water was added and the samples were reevaporated to dryness.

Standards were prepared by weighing 5 mg amounts and placing them in 2 dram vials.

Trimethylsilyle (TMS) derivatives were prepared by adding 1 ml of Catalyst-Solvent Mixture (Pierce Chemical Co.) to the 10 mg samples and the 5 mg standards. These mixtures were shaken vigorously for 30 sec and allowed to stand 5 min prior to injection into the gas chromatography. Silylized samples were stable for one week when stored at 4 C.

Analysis of silylized carbohydrates was accomplished using a Perkin-Elmer 990 gas chromatography with a flame ionization detector utilizing a 1.83 m by 0.32 cm spiral column packed with 3% OV-101 on Chromabsorb Q. The carrier gas was hydrogen used at a flow rate of 2.5 l/min. The injector port and the detector were operated at 300 C. Programmed runs were accomplished by injecting the sample under test, allowing a 1 min delay, and increasing the oven temperature at a rate of 12 C/min from an initial temperature of 160 C to a final temperature of 250 C. The detector range was set at X10 with attentuation settings of X8, X16, X32 or X64. Data were recorded using a Sargent-Welch

strip chart recorder operating at a speed of 0.5 in/min. Retention times and peak areas were determined from the charts for qualitative and quantitative evaluation, respectively.

Amino acid analysis

Samples at a concentration of 5 mg/ml were prepared as indicated in the section Hydrolytic Methods using 2 ml of constant boiling 6 N HCl at 100 C for 24, 48, or 72 h. Dried samples were dissolved in 2 ml of 0.2 M citrate buffer, pH 2.2, and a volume of 0.6 ml was chromatographed on a Beckman Spinco model 120 C amino acid analyzer according to the methods of Spackman, et al. (1958).

X-ray probe analysis

Cells or cysts were washed three times with distilled water and diluted to an optical density of 0.8 at 600 nm. One loopful of the suspension was placed on an aluminum stub, heat fixed, and coated with 20 Å of carbon in a Denton vacuum evaporation unit equipped with a rotary stage. Prepared stubs were placed in a JEOLCO scanning electron microscope equipped with a lithium-silicon detector. The specimen was scanned in the image mode to locate a desirable field containing well-isolated organisms. The area of choice was photographed and the instrument was transferred to the X-ray mode for calcium analysis.

Glycerol density gradients

Linear glycerol density gradients (8-33%) were prepared in 3 ml nitrocellulose tubes and layered with 0.1 ml of either sample or standard. Protein standards included aldolase, hemoglobin, catalase, chymotrypsinogen A, and ovalbumin. The prepared gradients

were centrifuged in a Beckman Spinco Model-L preparative ultracentrifuge at 39,000 rpm for 6 h at 4 C. Fractions of 0.5 ml volume were collected using a Buchler automatic fraction collector by puncturing the bottom of the tubes with a device allowing minimum disturbance of the sample. Protein standards were located by measurement of absorbance at 280 nm while LPS was located by the carbocyanin dye assay method.

Gel electrophoresis

Sodium dodecyl sulfate (SDS) gel electrophoresis was accomplished according to the method of Weber and Osborn (1969). Protein bands were located by the method of Chrombach, et al (1967) and carbohydrate bands were located by the method of Wardi and Michos (1972). Mobilities were determined by the formula:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

Mobilities were plotted against known molecular weight standards on a semi-logrithmic scale. Standards were aldolase, ribonuclease-A, chymotrypsinogen-A, and ovalbumin.

RESULTS

This investigation was conducted in four parts: (1) development and characterization of a suitable culture method for production of the large volumes of cysts required for chemical studies; (2) definitive examination of the morphogenesis of cells during encystment by SEM and TEM; (3) fractionation of cysts into their various components; and (4) partial chemical analysis of the cyst coat fractions.

Culture methods

Because of the divergent views of Stevenson and Socolofsky (1972) and Lin and Sadoff (1968) regarding the ability of Azotobacter to encyst in the presence of glucose, it was necessary for comparative purposes to reexamine the culture methods utilized by these investigators for production of cysts. The cultural conditions selected for evaluation included:

1. Burk's medium with 1% glucose.
2. Burk's medium with 1% glucose, periodically neutralized with 1 N KOH.
3. Burk's medium with 1% glucose and 0.6% powdered $CaCO_3$.
4. Burk's medium with 1% glucose and 0.6% $CaCO_3$ in a dialysis bag.
5. Burk's medium with 1% glucose, 0.1% BHB added at 18 h of growth (supplemented).
6. Burk's medium with 1% glucose, transferred to 0.1% BHB containing medium at 18 h (replacement).

A time course study of the pH of these cultures is presented in Figure 1. The pH of the control culture containing 1% glucose reached a minimum of 5.2 after 3 days of growth. When cultures were adjusted to the initial pH by addition of KOH, they rapidly returned to the lower pH typical of the control culture. The pH of these cultures remained in the neutral range only after the third daily addition of KOH. Cultures supplemented with 0.1% BHB at 18 h reached and maintained a minimum pH of 5.3 on the second day of growth. In the cultures grown in the presence of $CaCO_3$ in the dialysis bag, the pH declined initially but stabilized at 6.9 on the second day of growth and gradually increased to a pH of 7.4 on the fourth day of growth. The pH of these cultures decreased slightly to 7.2 by the sixth day of growth. Cultures produced by replacement of the glucose-containing medium with BHB-containing medium underwent an initial decline in pH to 6.8, after which the pH gradually reached a maximum of 7.7 on the fifth day of growth. Cultures produced in 1% glucose with 0.6% $CaCO_3$ underwent a similar initial decrease in pH, followed by an increase to pH 7.9 by the fourth day of growth.

Decreases in pH of the culture medium are attributed to the production of acid polysaccharides (Cohen and Johnstone, 1963). As the pH of the cultures decreased, the viscosity of cultural supernatants increased. The viscosities of the supernatants, which fall into three groups, are presented in Figure 2. The first group consists of supernatants from replacement and $CaCO_3$ cultures. In both cases, minimal viscosity increases were noted through 6 days of growth. The second group, consisting of cultures produced with $CaCO_3$ in a dialysis bag, and control cultures reached a

Fig. 1. pH of cultures of Azotobacter vinelandii produced by various cultural methods. Symbols: ●, culture buffered by addition of free $CaCO_3$; ■, culture buffered by use of $CaCO_3$ in a dialysis bag; ▲, control culture; ○, BHB-replacement culture; □, BHB-supplemented culture; △, KOH-neutralized culture.

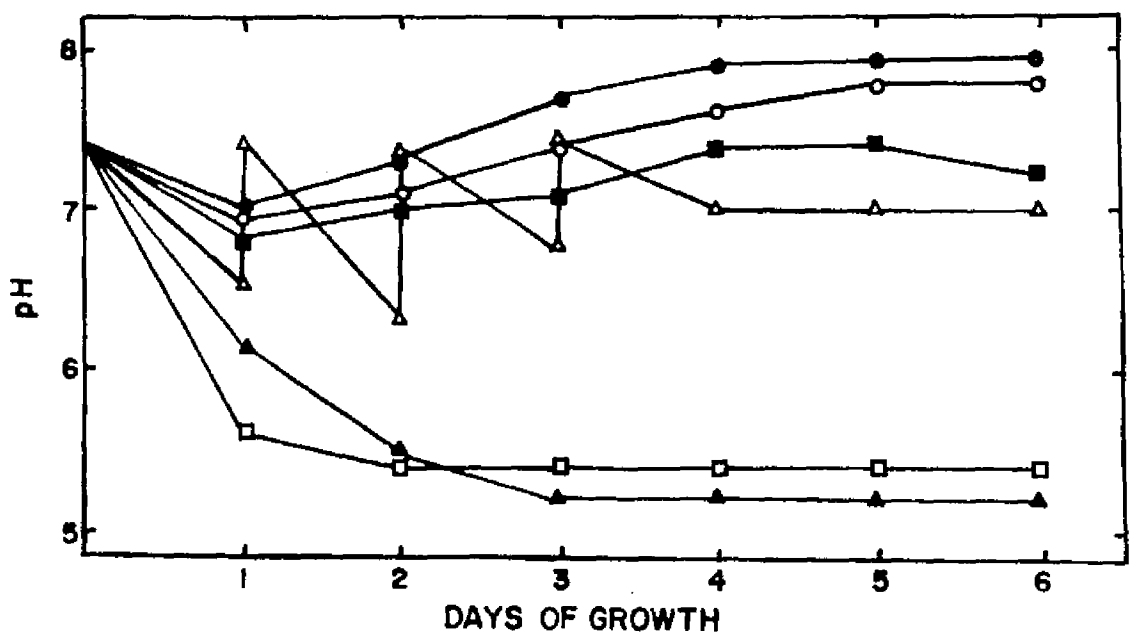
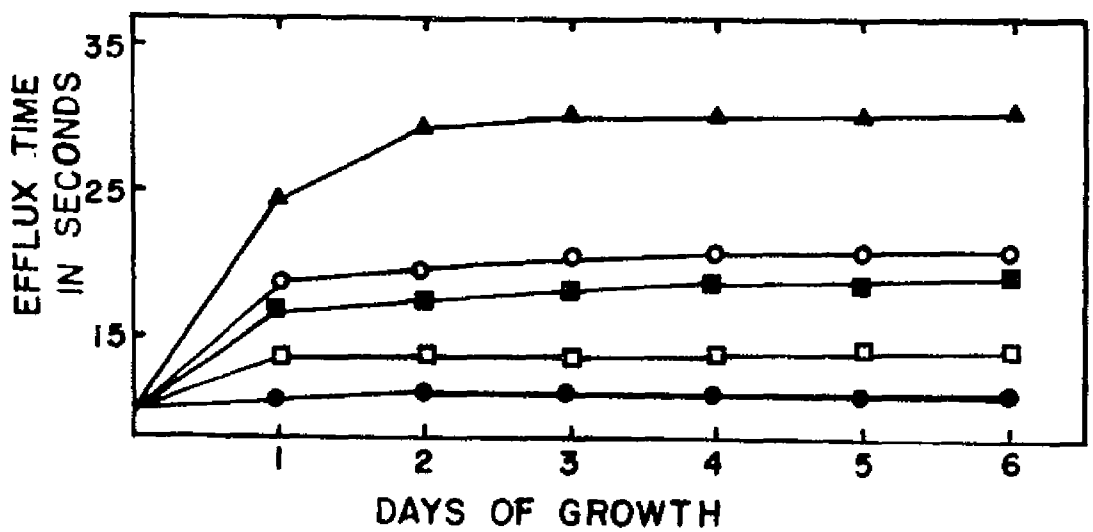

8
7
6
5
pH
1
2
3
4
5
6
DAYS OF GROWTH

Fig. 2. Viscosity of supernatants from cultures of Azotobacter vinelandii produced by various cultural methods. Symbols: ●, BHB-replacement culture; ■, culture buffered by use of $CaCO_3$ in a dialysis bag; ▲, KOH-neutralized culture; ○, control culture; □, culture buffered by addition of free $CaCO_3$.


EFFLUX TIME
IN SECONDS
35
25
15
1
2
3
4
5
6
DAYS OF GROWTH

moderate viscosity. The KOH-neutralized culture and the supplemental culture comprise the third group. Both of these cultures achieve high viscosities. The data from supplemented cultures is not included in Figure 2 because the efflux time after 1 day of growth was several hours. These cultures usually gelled when refrigerated at 4 C after 6 days of growth.

Cells produced by these culture methods were harvested by centrifugation and resuspended in Tris buffer, pH 8, for determination of lytic susceptibility as a criterion of encystment. Sensitivities to EDTA are presented in Figure 3. Nonencysting cells from the control culture exhibited no sensitivity to EDTA. Supplemental and KOH-neutralized cultures were not examined because phase microscopic analysis indicated complete absence of refractile bodies corresponding to mature cysts. Cysts produced in the presence of $CaCO_3$ in a dialysis bag were moderately sensitive to EDTA while cysts produced by replacement or by the addition of free $CaCO_3$ to the medium were markedly sensitive to EDTA, indicating rupture of the exine with release of the central body.

The efficiency of encystment compared to the amount of ethanol precipitable polysaccharide present in the culture supernatant is presented in Figure 4. Generally, when the percentage of encystment is high, the amount of polysaccharide in the culture supernatant remains low. Only cultures produced by replacement or by incorporation of free $CaCO_3$ into the culture medium encyst efficiently.

The extreme increases in viscosity occurring when cultures were supplemented with BHB prompted further investigation of these cultures. Phase microscopic observations indicated that the cells

Fig. 3. Lytic susceptibility of cysts produced by various cultural methods. Symbols: ●, culture buffered by addition of free $CaCO_3$; ■, BHB-replacement culture; ▲, control culture; ○, culture buffered by use of $CaCO_3$ in a dialysis bag.

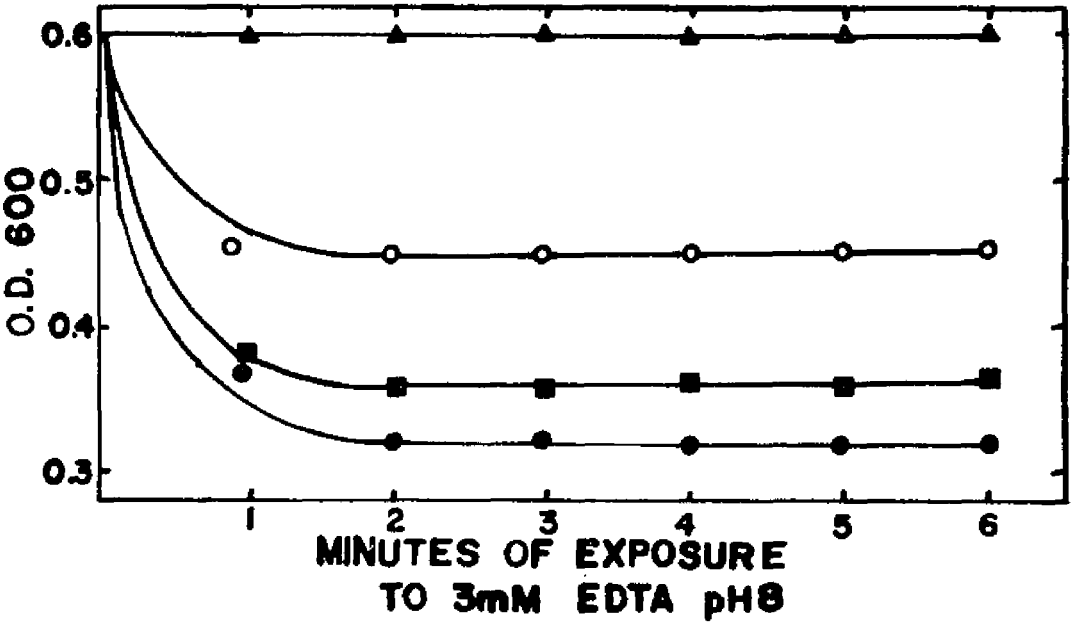
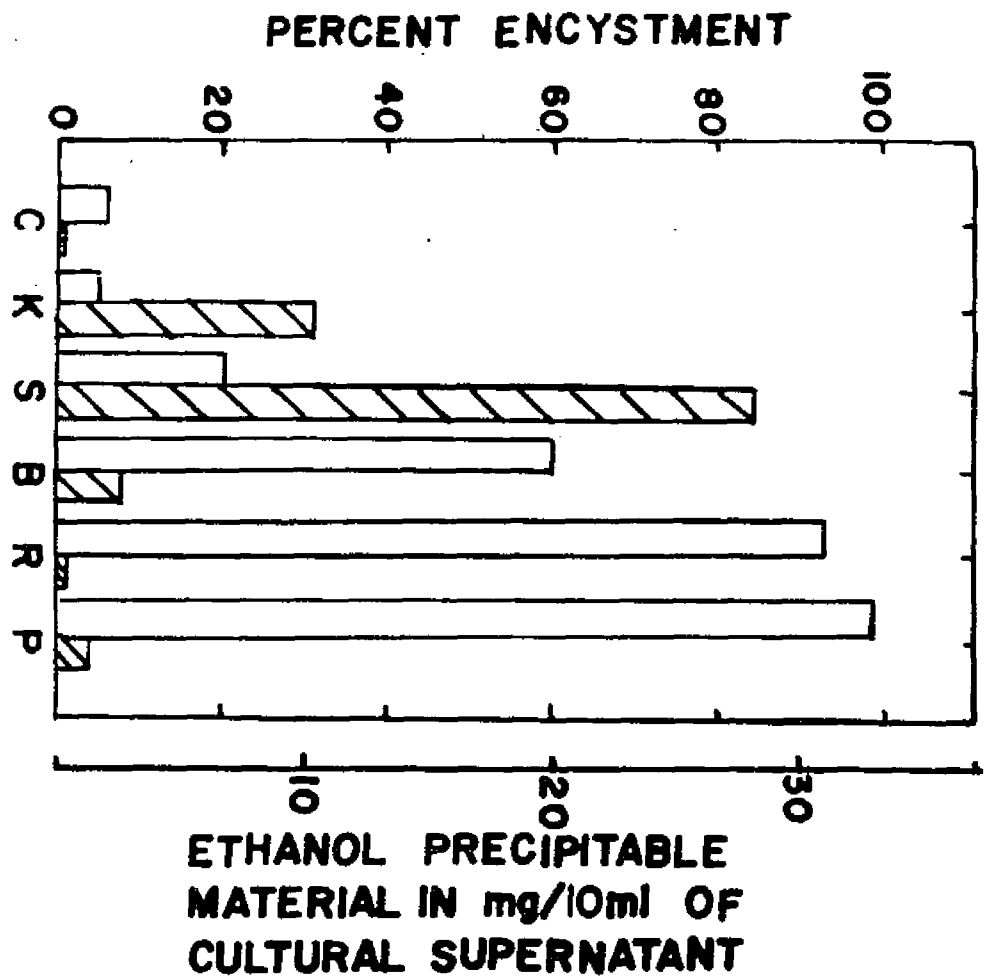

0.6
0.5
0.4
0.3
O.D. 600
1
2
3
4
5
6
MINUTES OF EXPOSURE
TO 3mM EDTA pH8

Fig. 4. Efficiency of encystment of Azotobacter vinelandii produced by various cultural methods as related to the amount of ethanol precipitable material in the culture supernatants. Symbols: ▭, percent encystment; ▨, mg/10ml of ethanol precipitable material; C, control culture; K, KOH-neutralized culture; S, BHB-supplemented culture; B, culture buffered by use of $CaCO_3$ in a dialysis bag; R, BHB-replacement culture; P, culture buffered by addition of free $CaCO_3$.

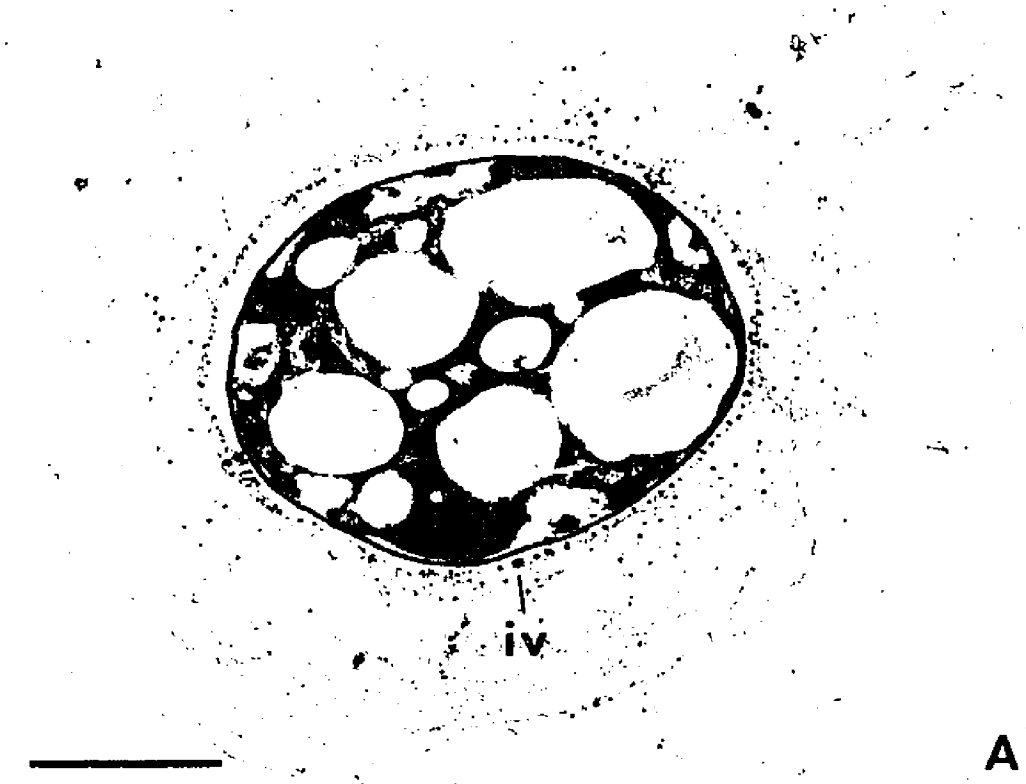

PERCENT ENCYSTMENT
0
20
40
60
80
100
C
K
S
B
R
P
10
20
30
ETHANOL PRECIPITABLE
MATERIAL IN mg/10ml OF
CULTURAL SUPERNATANT

had rounded-up and accumulated large quantities of PHB, but no cyst coat structures were evident. Loosely-associated fibrous elements resembling exine were evident in a free form throughout the preparation.

Electron microscopic observations of these cultures confirmed the results of phase microscopy concerning the accumulation of PHB by these cells (Figure 5A). The cells were surrounded by a gel-like structure containing numerous spherical vesicles. This outer structure appeared similar to the exine of fragile cysts reported by Vela and Cagle (1969). Free exine-like components from these cultures appeared as loosely-associated fibrous aggregates, somewhat more electron dense than the surrounding capsular polysaccharide (Figure 5B). Vesicles appeared throughout this structure and free in the surrounding areas.

The culture supernatants from BHB-supplemented cultures after four days of growth were divided into 100 ml aliquots and precipitated by the methods presented in Figure 6. The various amounts of precipitated materials produced by exposure to the different methods are thought to represent selective precipitation of protein, polysaccharide and/or LPS. The proposed composition of these fractions is presented in Table 3. The extreme increase in viscosity of the culture supernatants is apparently the result of excretion of a LPS-LP complex which in turn complexes with the capsular polysaccharide normally present. Since $CaCl_2$ readily precipitates the total complex, the absence of sufficient calcium in the culture medium enables the complex to remain in a soluble form.

Fig. 5. Electron micrographs of thin sections of Azotobacter vinelandii prepared from BHB-supplemented cultures. A. Central body of cell undergoing abortive encystment surrounded by incomplete fragments of exine containing numerous intine vesicles (iv). B. Exine-like aggregate appearing free of central body, but containing numerous intine vesicles (iv). The markers represent 1 μm.


iv
A

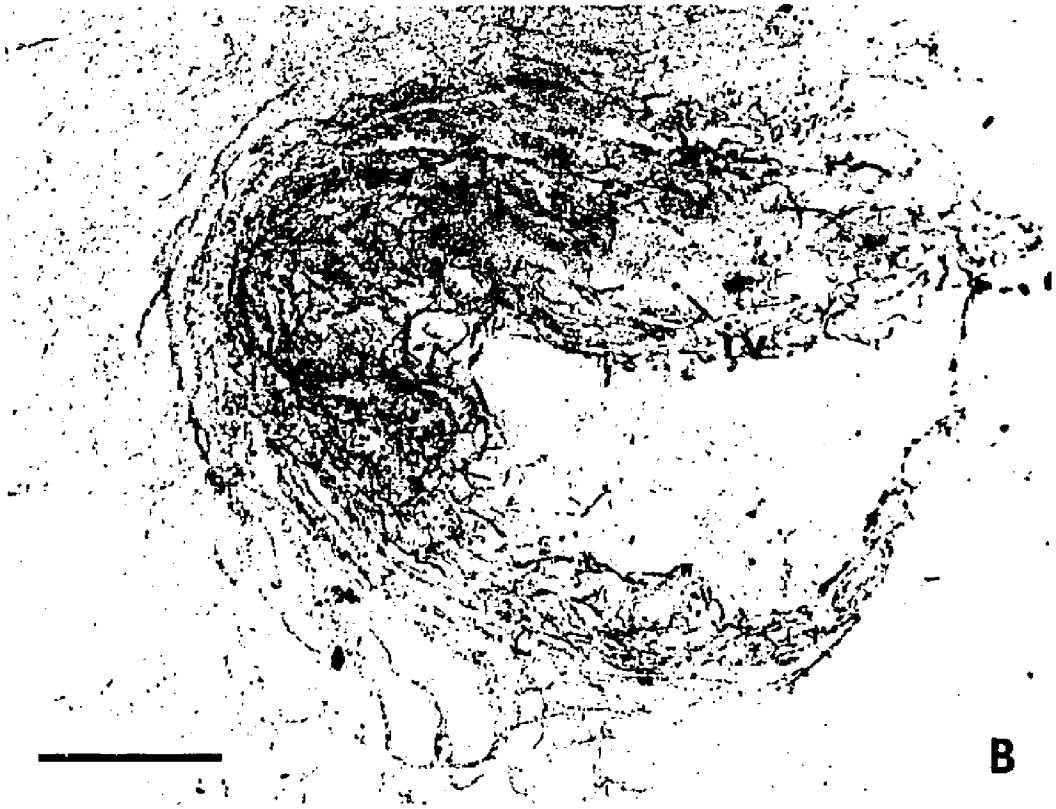

B

Fig. 6. Effect of the method of precipitation on the yield of precipitable materials from cultural supernatants of BHB-supplemented cultures.

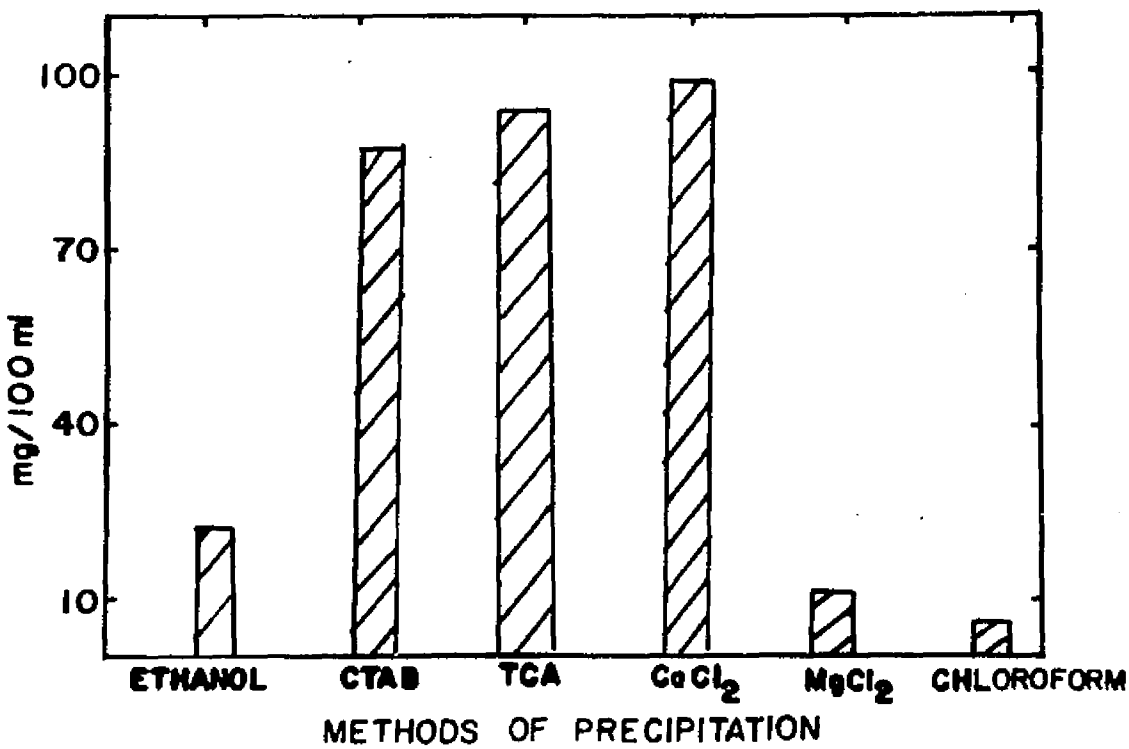
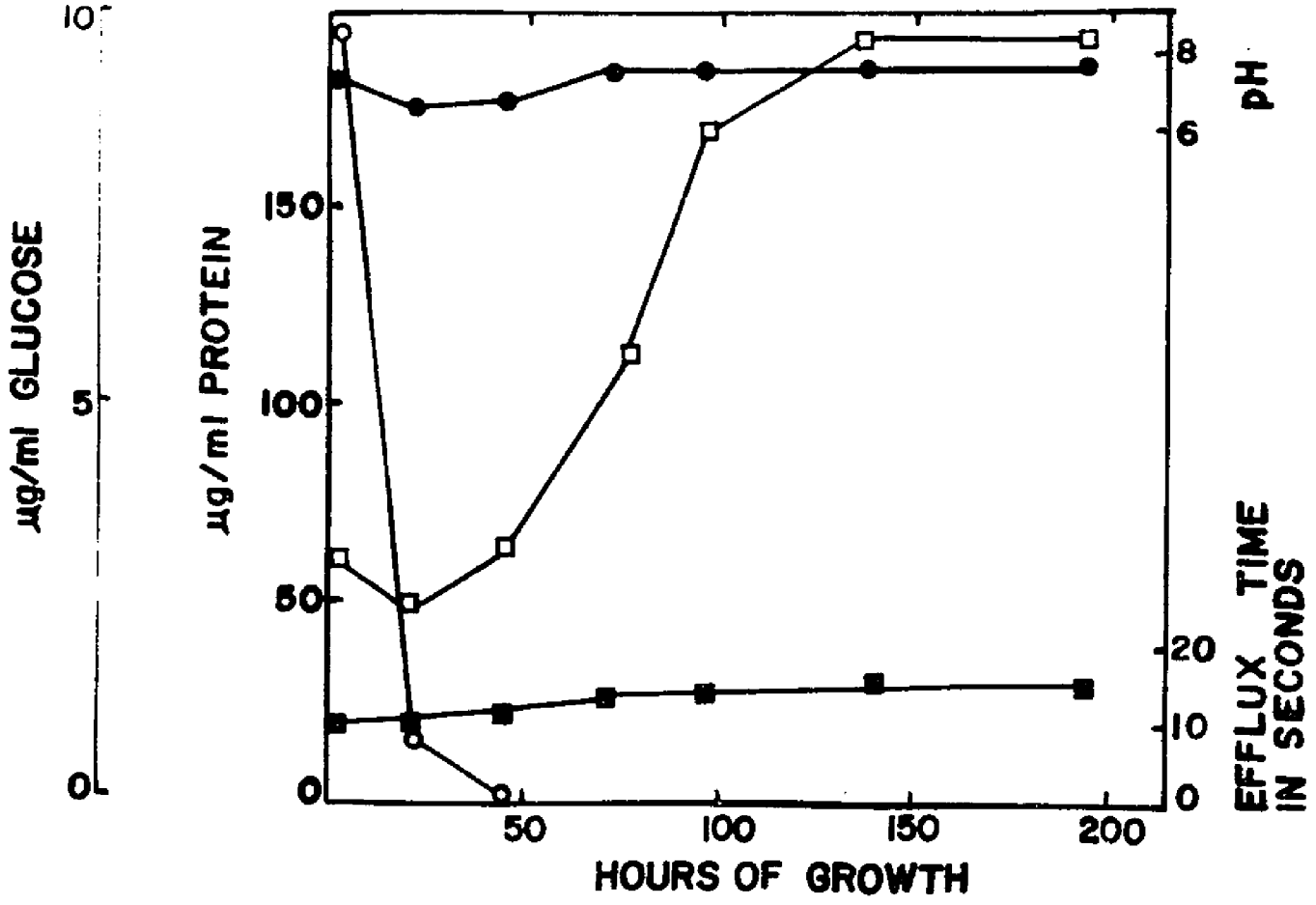

100
70
40
10
mg/100 ml
ETHANOL
CTAB
TCA
CaCl2
MgCl2
CHLOROFORM
METHODS OF PRECIPITATION

Table 3. Composition of culture supernatants from cells produced by the BHB-supplemental method.

Precipitant	Reference	Proposed fraction identification	Yield, mg/100ml
Chloroform	Knox, et al., 1966	Protein	6
$MgCl_2$	Westphal, et al., 1952	LPS	11
Ethanol	Lin and Sadoff, 1969	LPS-Protein	22
CTAB	Davis and Clapp, 1961	Polysaccharide	87
TCA	Lin and Sadoff, 1969	Polysaccharide-Protein	93
$CaCl_2$	Stevenson and Socolofsky, 1967	Polysaccharide-LPS-Protein	98

Since incorporation of 0.6% powdered $CaCO_3$ into the glucose-containing culture medium resulted in encystment of 98% of the cells, this method was chosen as the basis for development of a batch culture technique for production of the large quantities of cysts required for analytical studies. For batch cultures, 12 l of Burk's nitrogen-free medium, pH 7.4, was autoclaved in a 15 l fermentor vessel. The vessel was placed in the water bath of a New Brunswick fermentor at 32 C and stirred at 180 rpm for 2 days to redissolve the salts which had precipitated during sterilization of the medium. Glucose, containing powdered $CaCO_3$, was added to a final concentration of 1% and 0.6%, respectively. The fermentor vessel was inoculated by addition of a volume of 18 h vegetative cells equal to 10% of the final volume. The antifoam control was connected and the culture was incubated at 32 C with the sparger operating at 180 rpm and aeration at a rate of 2 l/min (Dalton and Postgate, 1969a; 1969b). Samples were withdrawn at 12 h intervals for 8 days. The cells were processed for TEM and the culture supernatants were analyzed according to the parameters presented in Figure 7. The pH of the fermentor culture essentially duplicates that of shake cultures buffered with $CaCO_3$. Glucose was rapidly exhausted during the first 36 h of growth. The presence of protein in the culture supernatant served as a guide to cultural development. Viscosity of the cultural supernatant remained low, indicating the presence of little free polysaccharide. Phase microscopic observations of the cells from the batch cultures indicated that encystment was complete in 4 days.

Fig. 7. Parameters of growth monitored during encystment of Azotobacter vinelandii in batch culture. Symbols: ●, pH; ■, viscosity; ○, glucose; □, protein.


µg/ml GLUCOSE
10
5
0
µg/ml PROTEIN
150
100
50
0
pH
8
6
EFFLUX TIME IN SECONDS
20
10
0
50
100
150
200
HOURS OF GROWTH

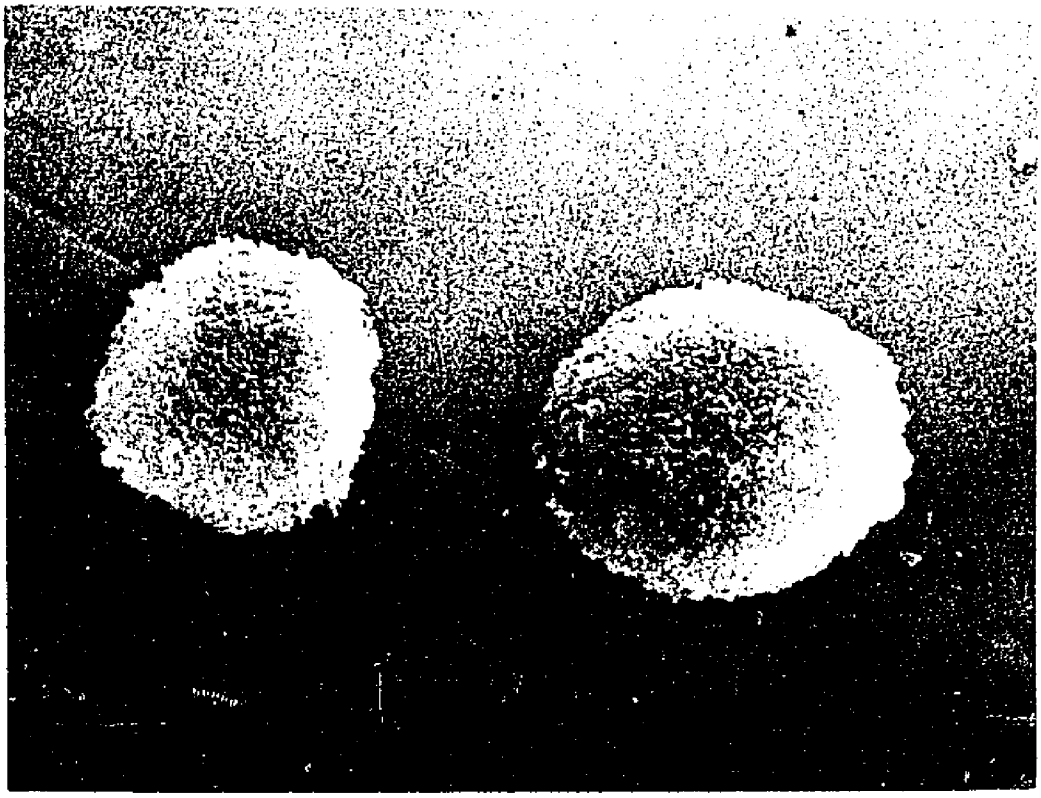
Morphogenesis

Scanning electron microscopic study

Intact colonies of Azotobacter vinelandii were prepared for scanning electron microscopy by critical point drying with liquid CO_2. Colonies of 24 h cells were convex and somewhat irregularly shaped (Figure 8). Individual cells appeared as short, plump rods in various stages of cell division. Colonies examined after 48 h of growth consisted of ovoid cells completely covered with a thick layer of capsular polysaccharide which obscured all details of the individual cells (Figure 9). In order to facilitate viewing of individual cells in situ, the crowns of the colonies were removed by gentle agitation in the first step of the dehydration series. Individual cells within a colony after 24 h of growth are presented in Figure 10A. The rod-shaped, peritrichously flagellated cells were covered with numerous papular outpouchings, appearing as light nodules on the surface of the cells. By 48 h (Figure 10B), the cells were ovoid and flagella appeared wrapped around the cells, partially occluded by capsular polysaccharide. Further accumulations of capsular polysaccharide, appearing as a fibrous network with multiple points of attachment to the cells, were noted after 72 h of growth (Figure 11A). Flagella were no longer evident at 72 h. No further changes were apparent through 6 days of growth (Figure 11B).

Since image properties of the scanning electron microscope depend upon topography and elemental composition of the specimen, it was of interest to examine a colony of cysts in an uncoated preparation. The image formed appears in Figure 12A. The cysts were spherical and possessed a dark central area, mottled with lighter areas, corresponding

Fig. 8. Scanning electron micrographs of intact colonies of Azotobacter vinelandii. A. Colonies of vegetative cells after 24 h of growth. B. Rod-shaped cells in active cell division. The markers represent 20 μm.



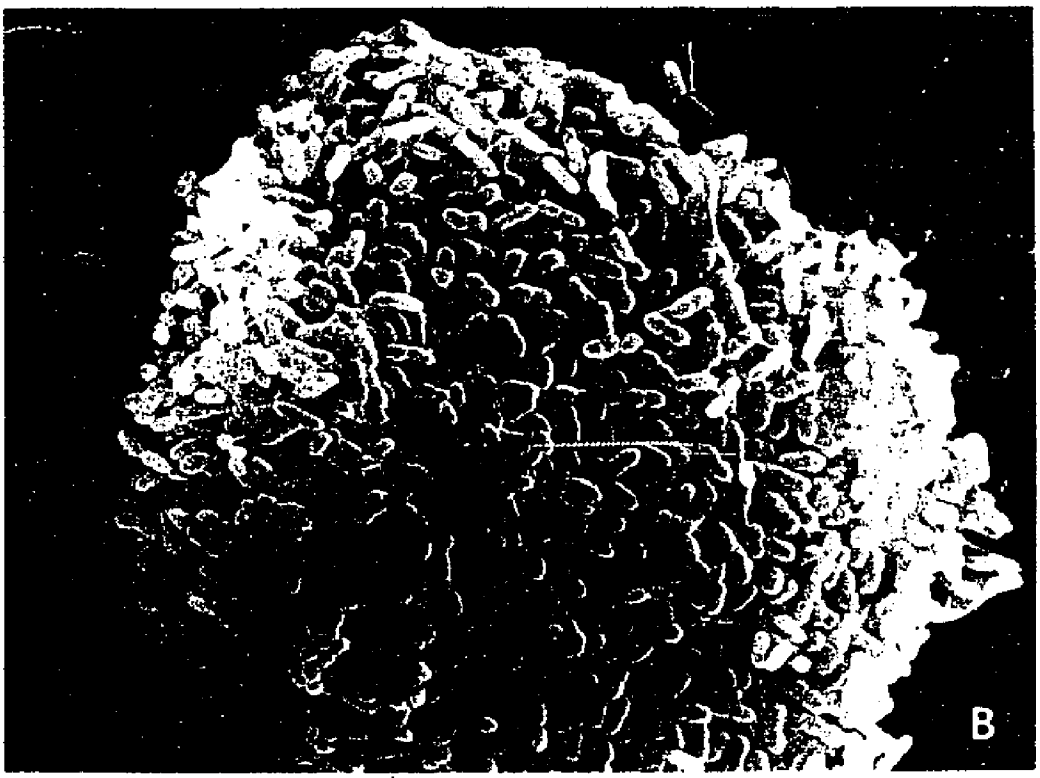
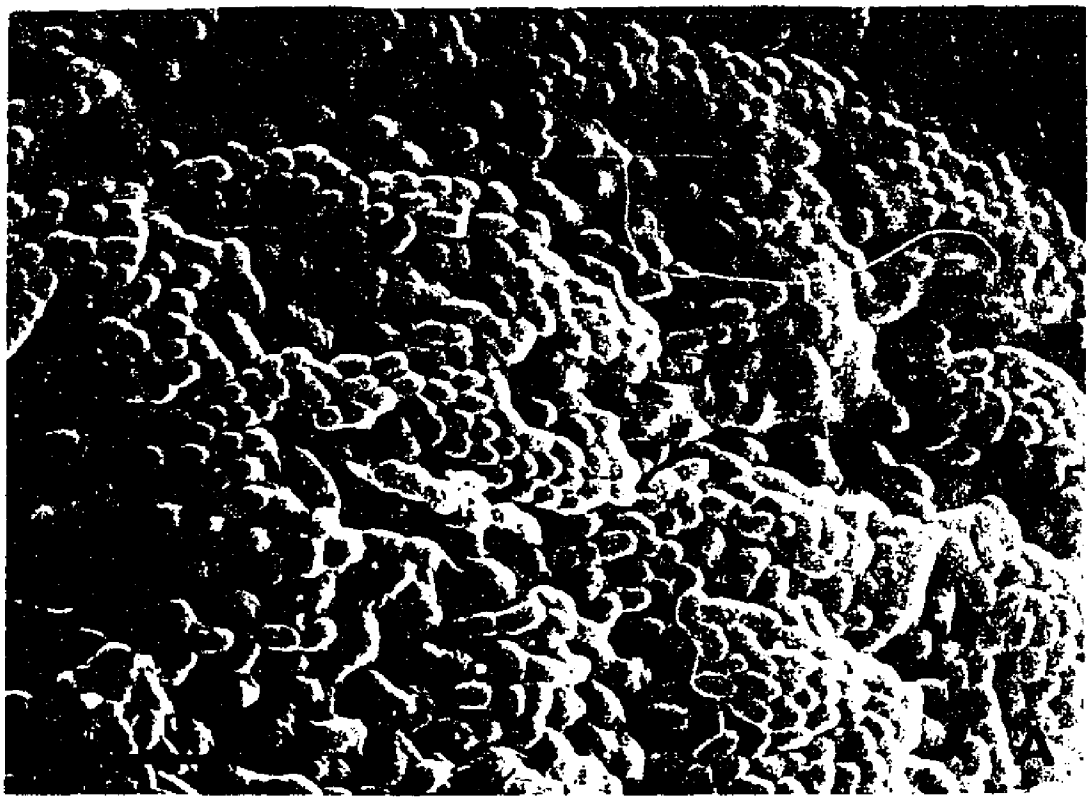

B

Fig. 9. Scanning electron micrographs of intact colonies of Azotobacter vinelandii demonstrating the copious capsular polysaccharide. A. Precystic cells after 48 h of growth. B. Edge of a colony demonstrating the masking effect of the capsular polysaccharide. The markers represent 10 μm.



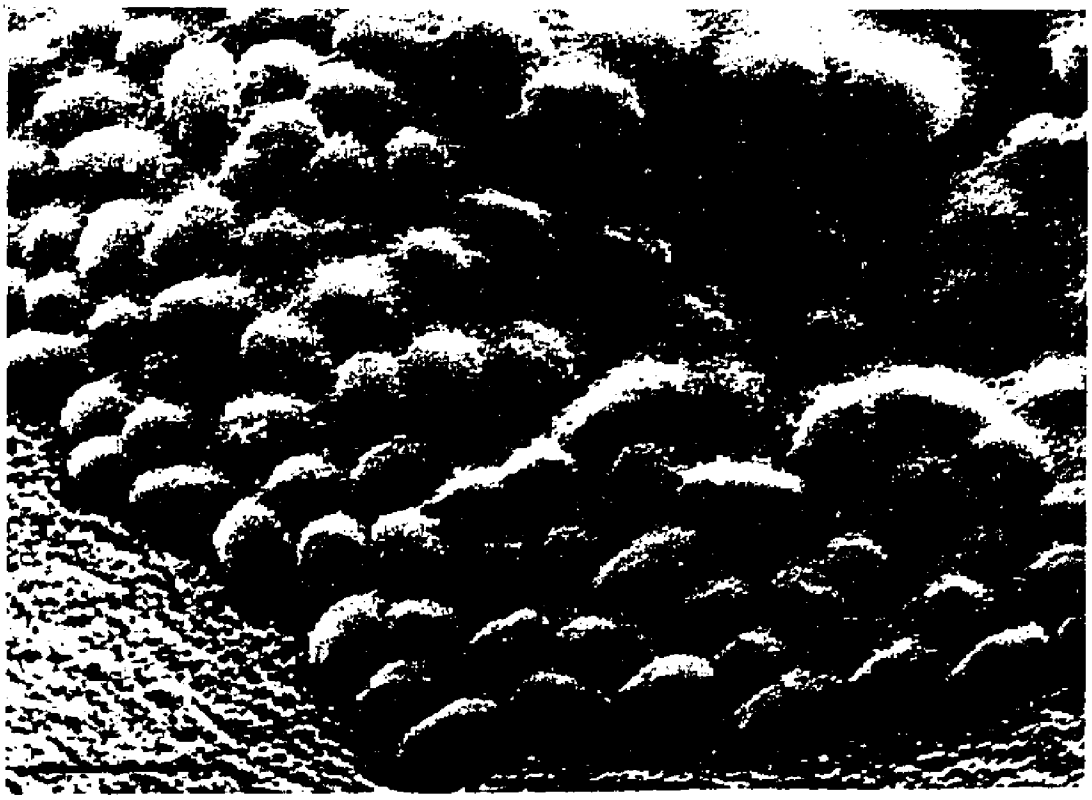
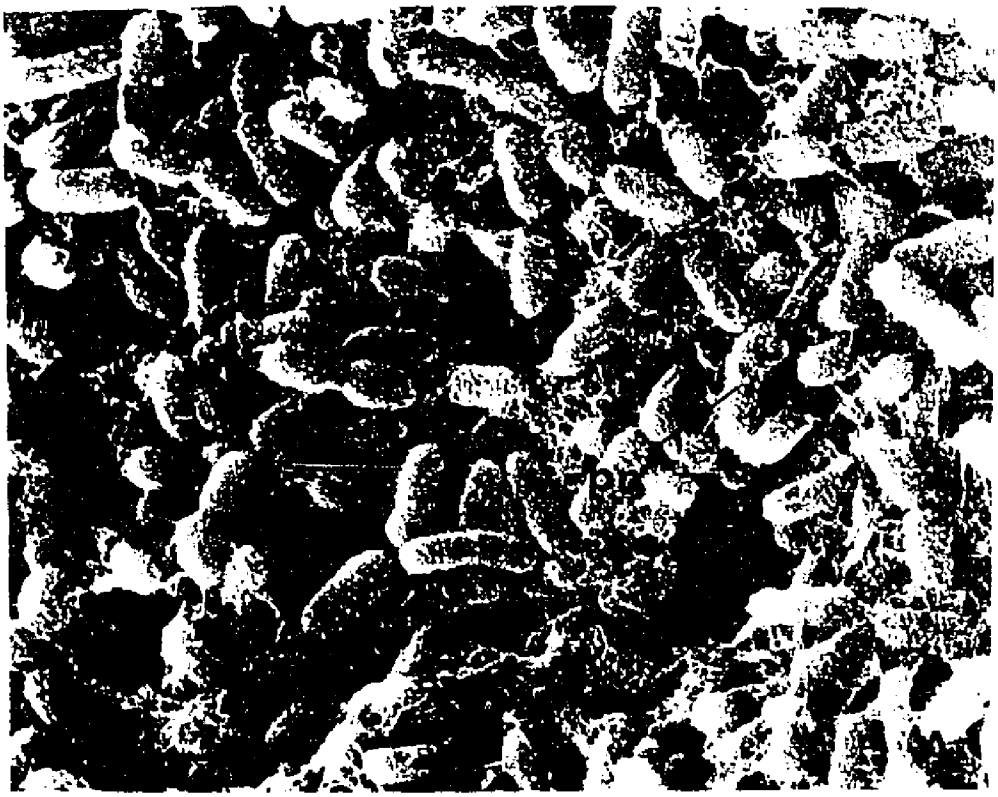


Fig. 10. Scanning electron micrographs of vegetative cells of Azotobacter vinelandii during encystment. A. Peritrichously flagellated cells after 24 h of growth. Papular nodules (pn) appear on the surface of the cells. B. Precystic cells after 48 h of growth. Flagella (f) appear wrapped around the cells. The markers represent 5 μm.



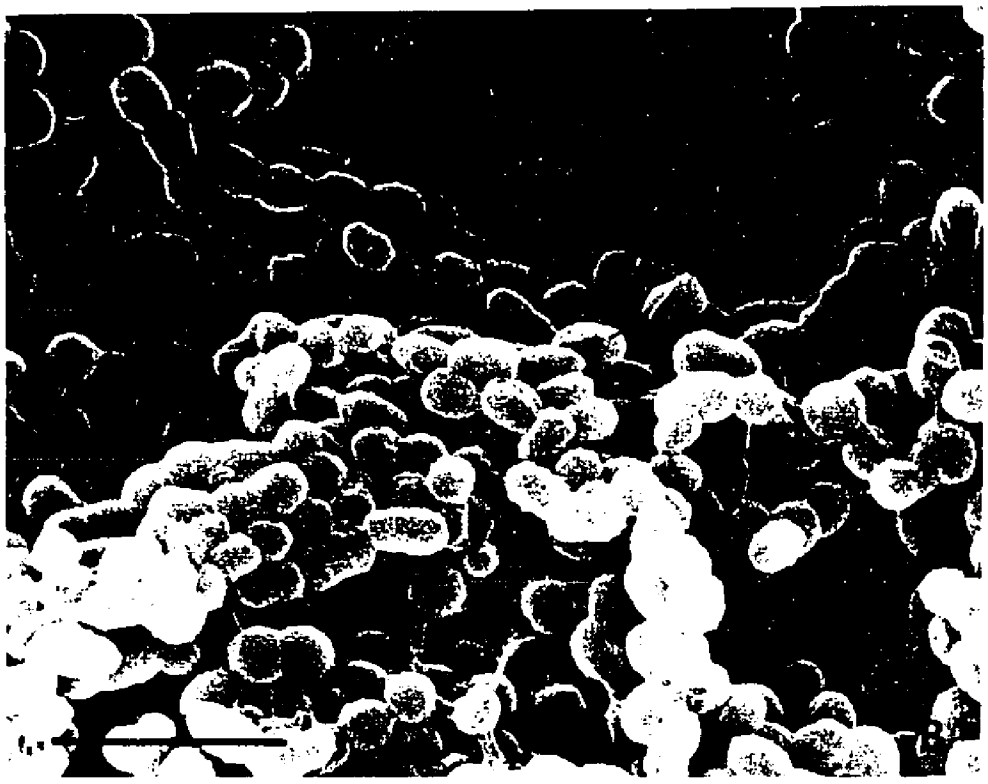
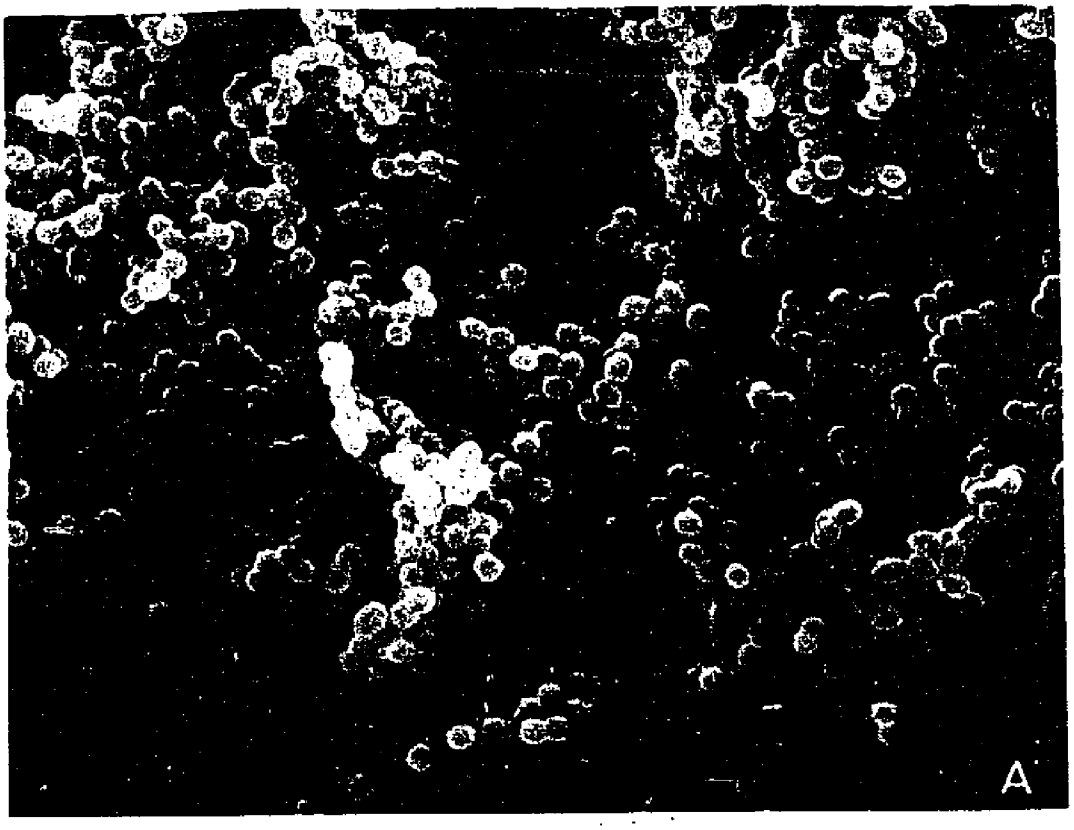
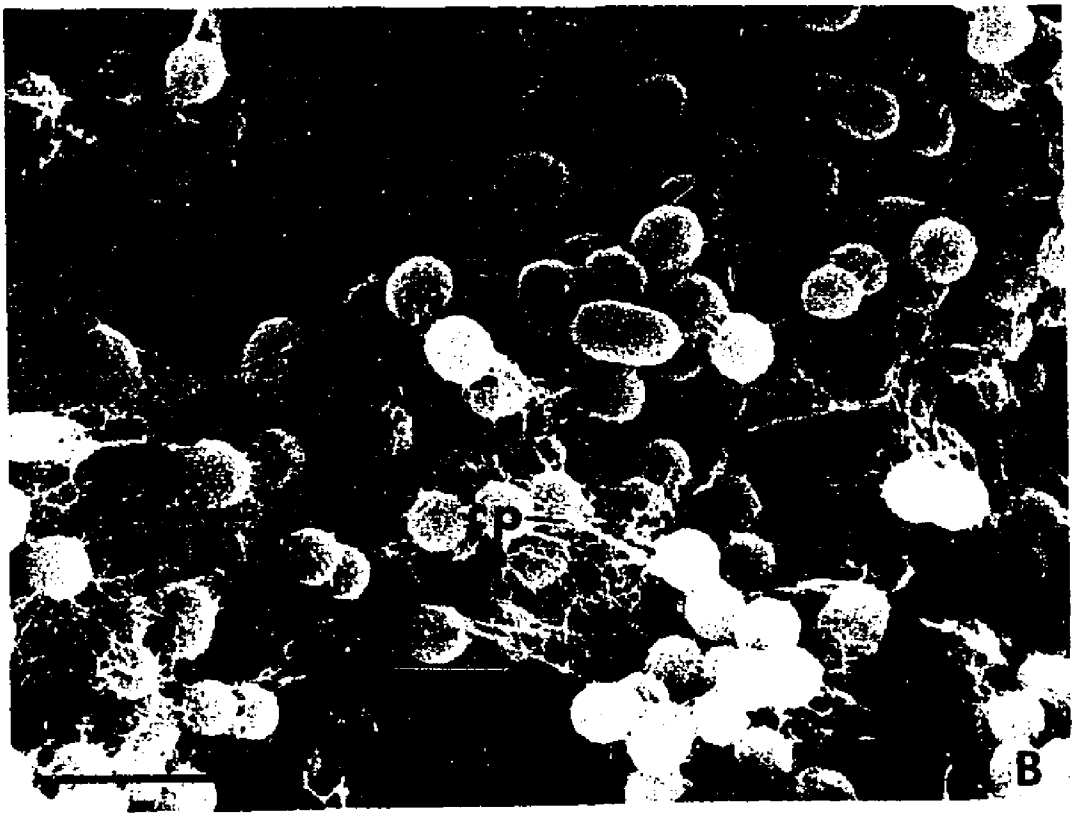


Fig. 11. Scanning electron micrographs of cysts of Azotobacter vinelandii. A. Cysts after 72 h of growth. B. Cysts after 96 h of growth. Capsular polysaccharide (cp) appears as a fibrous network surrounding the cysts. The markers represent 5 μm.


A

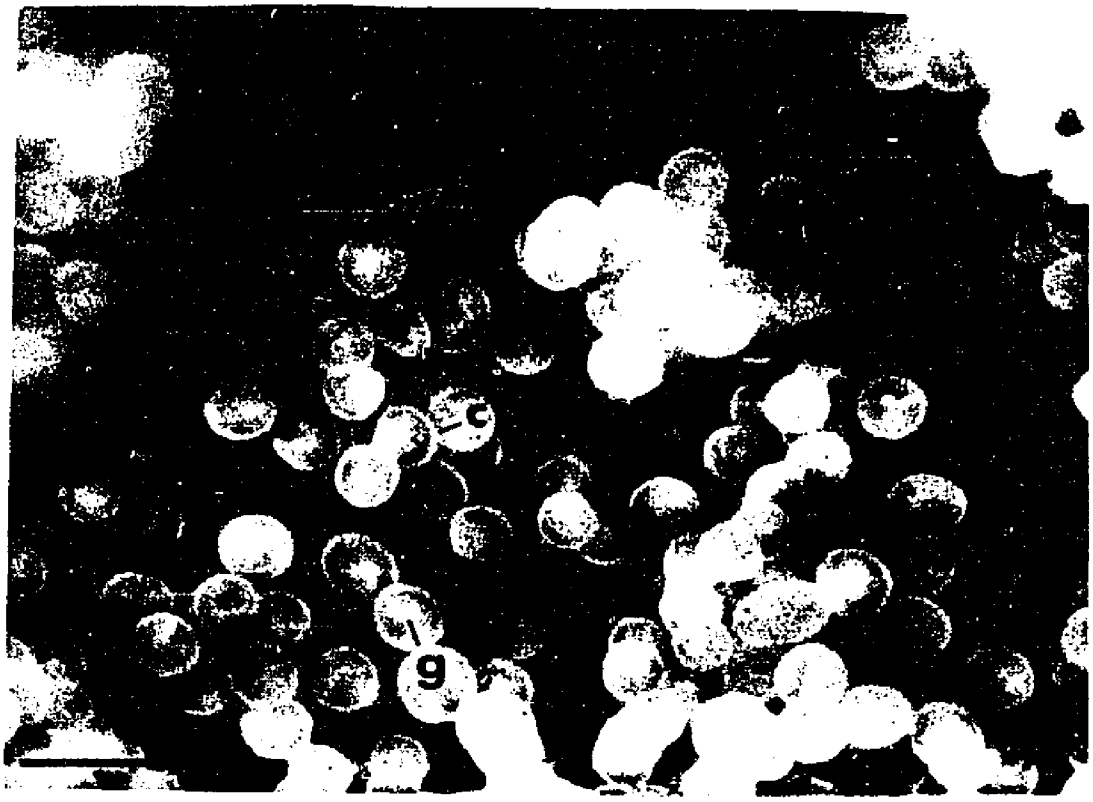

P
B

to the central body with numerous PHB granules. The cyst coat was evident as a light ring surrounding the cyst. No evidence of capsular polysaccharide was apparent. This image of a cyst corresponds directly to the thin section of a cyst presented in Figure 12B. The central body was surrounded by the typical cell wall of the vegetative cell. PHB granules appeared as electron transparent areas in the cytoplasm of the central body. The cyst coat appeared as a continuous, dense, fibrous network surrounded on the exterior by a less-dense layer of capsular polysaccharide. The spatial and structural agreement observed indicates that critical point drying is an effective method for preservation of cells or cysts of Azotobacter vinelandii.

Transmission electron microscopic study

Transmission electron microscopic preparations, particularly ultrathin sections, are especially subject to the production of artifacts during dehydration, fixation, embedding, or sectioning. The work of early investigators was, therefore, of limited value for assessment of the sequential development of cells during encystment. A cyst fixed by the $KMnO_4$ method of Luft (1956), is presented in Figure 13A. This stylized rendition of the cyst was considered a classical representation until only a few yeare ago. The drastic effect of $KMnO_4$ as an oxidizing agent has obliterated most of the structural detail of the cytoplasm and the cyst coat; however, a few spherical vesicles are evident. The capsule external to the cyst coat is practically destroyed. When cysts were fixed with glutaraldehyde, the effects of shrinkage were evident in Figure 13B. Ribosome structure of the central body is preserved with greater fidelity than with $KMnO_4$ fixation. The intine appears similar to $KMnO_4$ fixed

Fig. 12. Scanning electron micrograph of cysts of Azotobacter vinelandii in an uncoated specimen compared to a thin section of a cyst viewed by transmission electron microscopy. A. Uncoated cysts from a 96 h culture. Cyst coat (c) appears as a light ring around the cells. PHB granules (g) appear as light areas within the central body. The marker represents 5 μm. B. Thin section of a cyst at 72 h of growth. The central body (cb) contains numerous PHB granules (g). The cyst coat (c) appears fibrous. Intine vesicles (iv) appear at the junction of the cyst coat and the central body cell wall (cw). The losely-associated material external to the cyst coat is capsular polysaccharide (cp). The marker represents 1 μm.


g
g

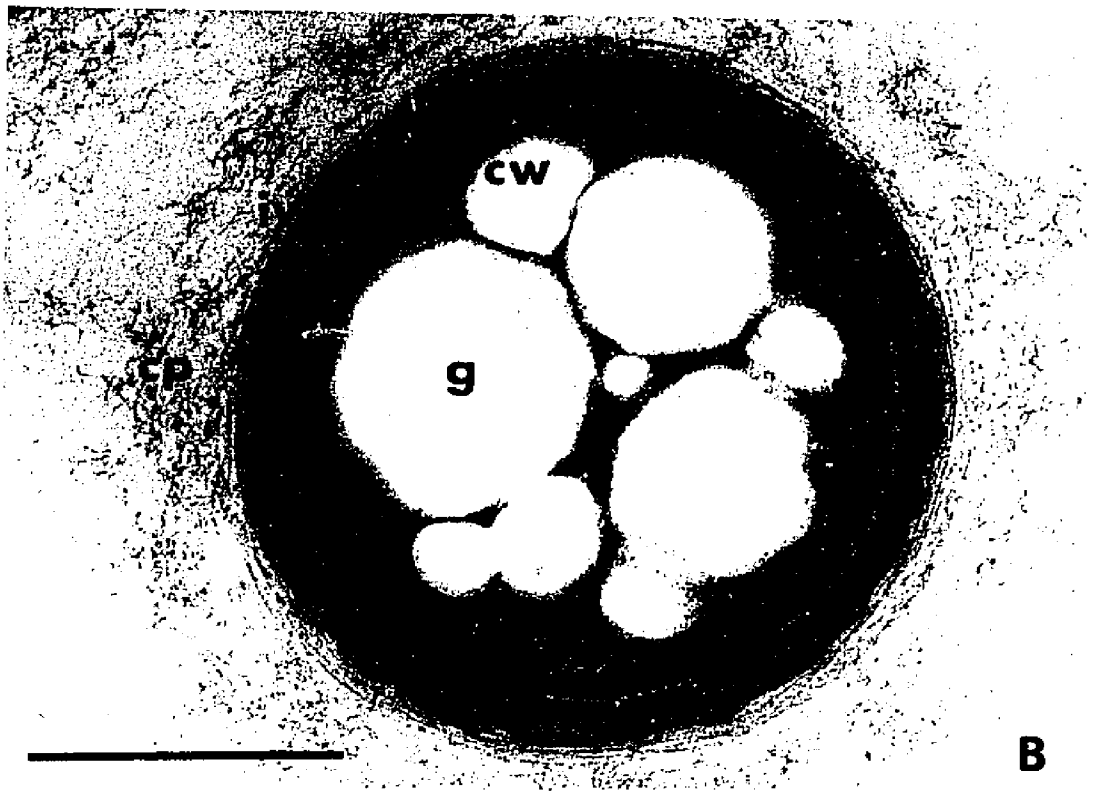
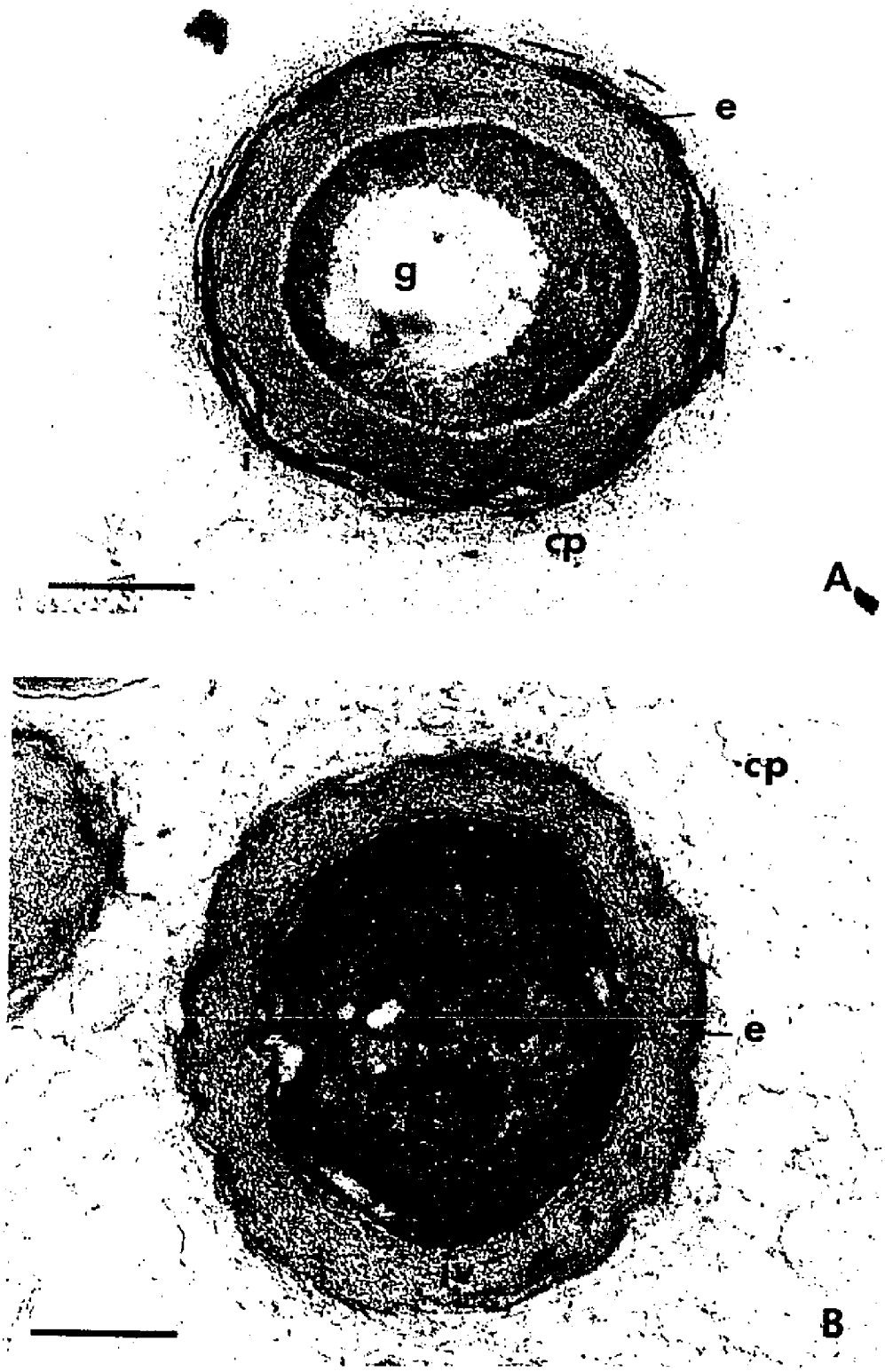

cw
g
cp
B

Fig. 13. Cysts of Azotobacter vinelandii in thin sections prepared for transmission electron microscopy by $KMnO_4$ or glutaraldehyde fixation. A. $KMnO_4$-fixed specimen demonstrating the dense exine (e), fibrous intine (i), intine vesicles (iv), PHB granules (g) and central body (cb) of the mature cyst. Capsular polysaccharide (cp) was poorly preserved. B. Glutaraldehyde-fixed specimen demonstrating the exine (e) intine (i), intine vesicles (iv), central body (cb) and capsular polysaccharide (cp) of a mature cyst. The markers represent 0.5 μm.

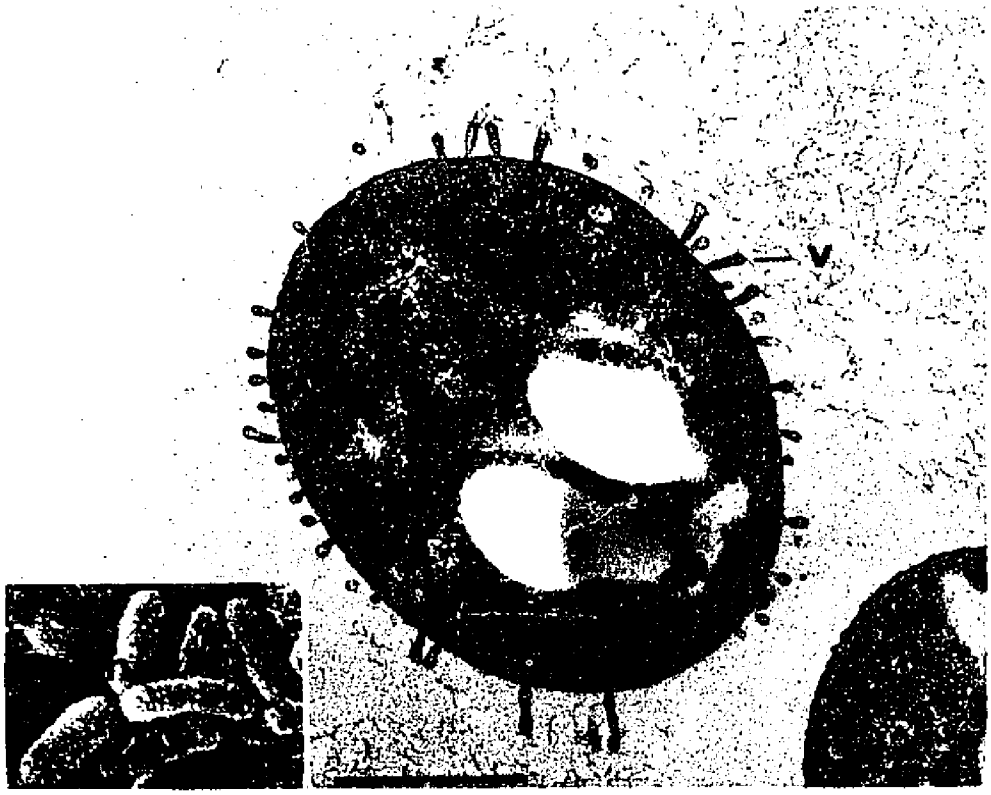

e
g
cp
A
cp
e
B

cysts, but the exine appears completely different, lacking the dense bark-like outer layer. Capsular polysaccharide is somewhat better preserved. The possibilities of artifact production caused investigators to use extreme caution when making interpretations from micrographs of these types.

Since the sequential ultrastructural alterations associated with encystment are dependent upon a method of fixation which results in minimal alteration of the cell, preliminary experiments were conducted to determine the most effective method of fixation. The method of choice consisted of a double-fixation using glutaraldehyde and OsO_4 in the presence of the specific stain for acid polysaccharides, ruthenium red. This method was used exclusively for the remaining specimens.

Samples for morphogenic studies were withdrawn from fermentor cultures at 12 h intervals. The first ultrastructural alteration associated with encystment was the accumulation of PHB (Figure 14A). The second event was the occurrence of vesicular evaginations from the L-layer of the cell wall as early as 12 h after inoculation. These vesicles are believed to correspond to the papular nodules seen in SEM preparations of young vegetative cells (Figure 14B). The vesicles pinch off and remain in the capsular polysaccharide immediately external to the cell wall (Figure 14C). By 24 h, the first dense fibrous elements of the cyst coat appear immediately external to the vesicles (Figure 15A). These dense areas continue to accumulate in layers around the cell from 12 to 24 h (Figure 15B). By 36 h, these layers of cyst coat materials have reached about one half of their potential thickness (Figure 16A). No further evaginations occur

Fig. 14. Production of vesicles by vegetative cells of Azotobacter vinelandii examined in thin sections after 12 h of growth. A. Vesicles (v) evaginate from the L-layer of the cell wall. The marker represents 0.5 μm. B. The nodules (n) observed by SEM correspond to the vesicles appearing in thin sections. The marker represents 1 μm. C. The vesicles (v) pinch off and remain in the capsular polysaccharide (cp) adjacent to the cell. The marker represents 0.5 μm.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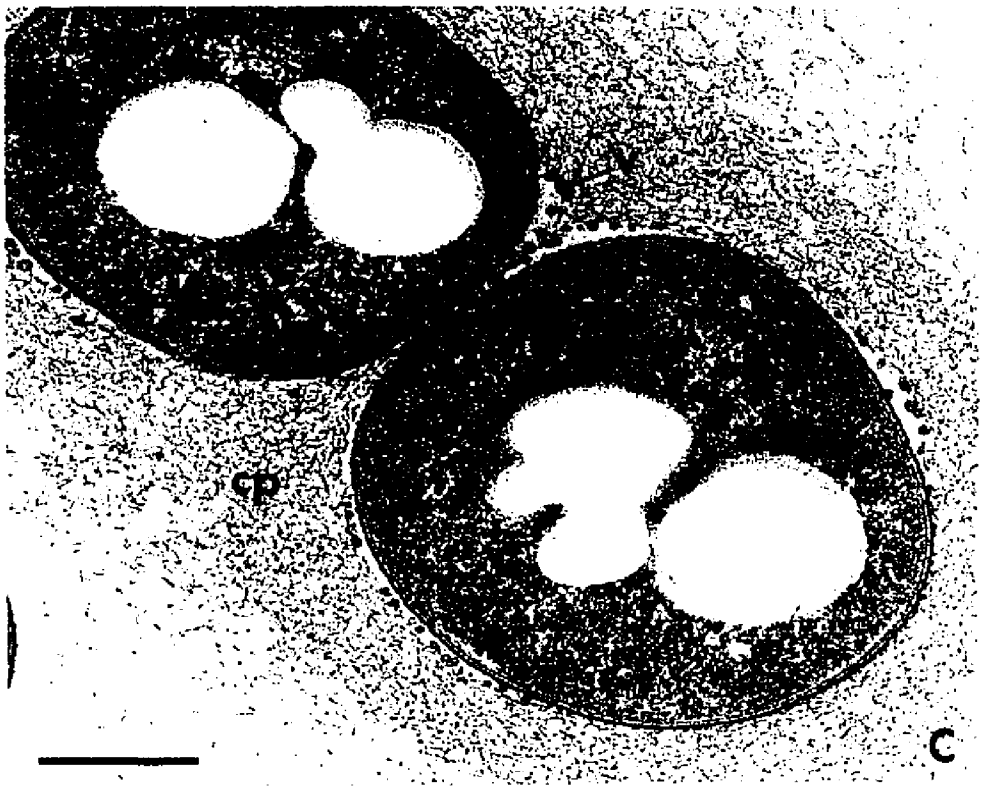
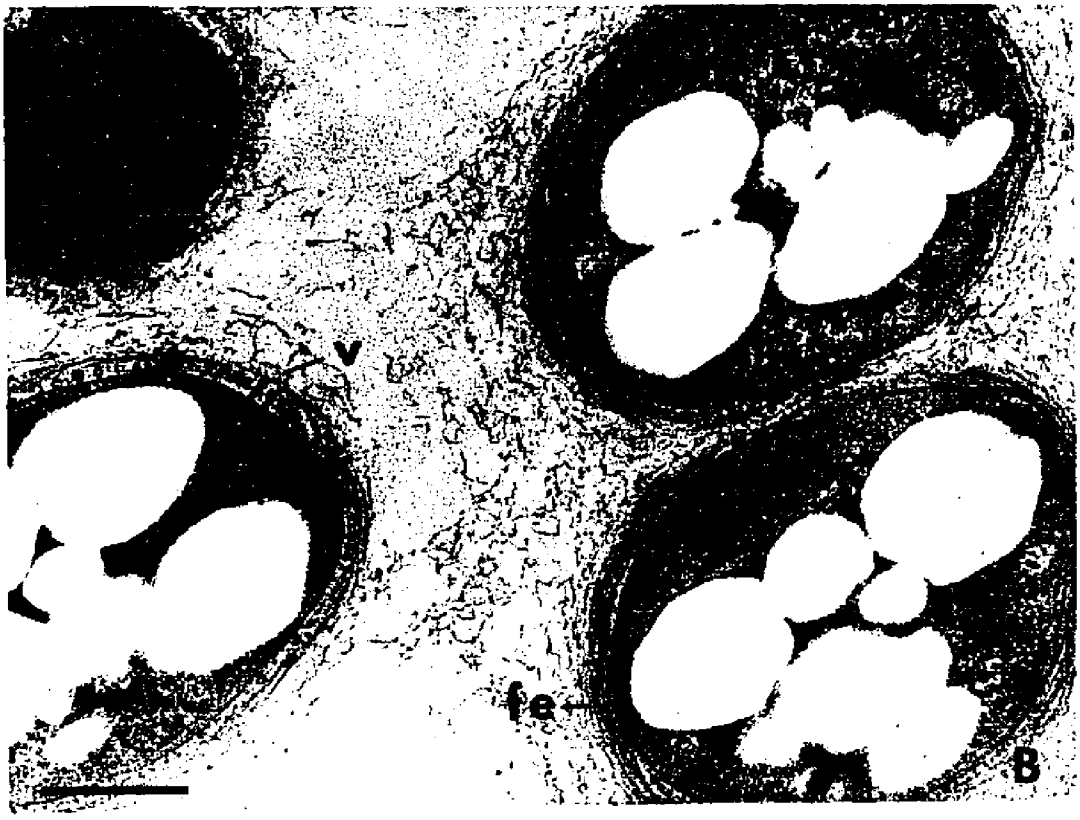

CP
C

Fig. 15. Morphogenesis of Azotobacter. Electron micrographs of thin sections of cells of Azotobacter vinelandii sampled at 24 h of growth. A. Fibrous elements (fe) of cyst coat begin to form. B. The fibrous elements (fe) continue to accumulate to form the layered structure characteristic of the cyst coat. Vesicles (v) continue to evaginate. The markers represent 0.5 μm.

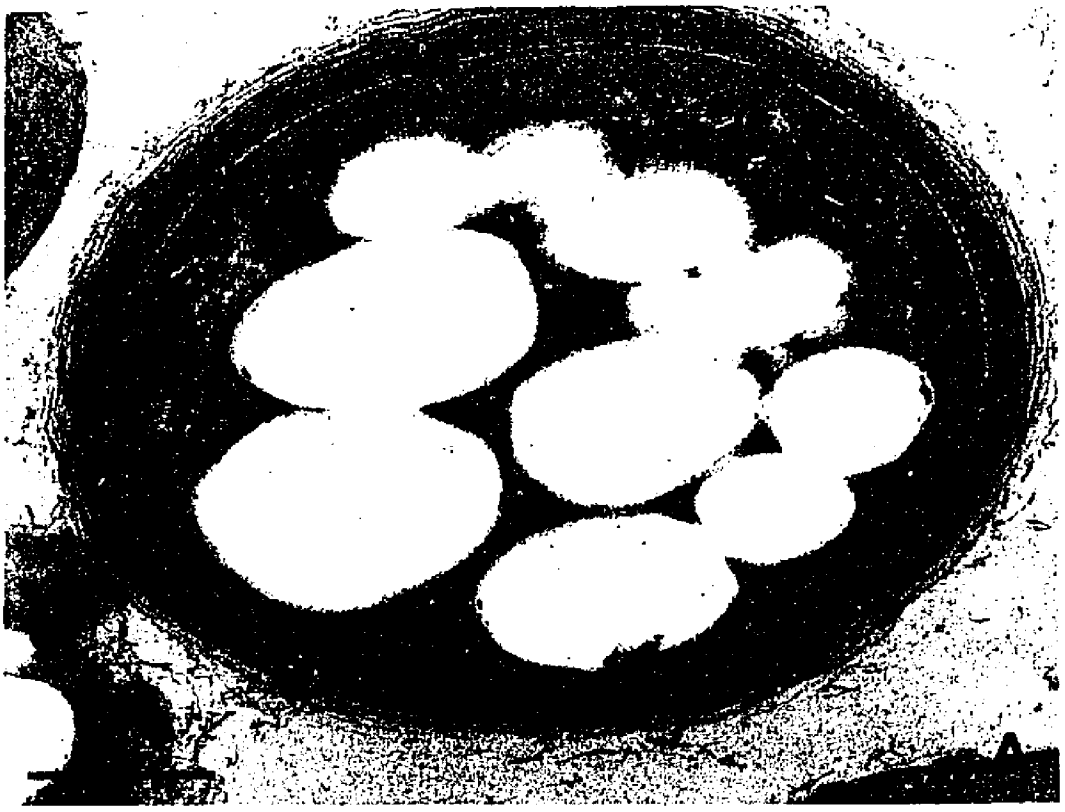

fe
A


v
fe
B

although vesicles remain visible along the cell wall of the central body and in cyst coat layers. At 48 h, the cyst coat appears differentiated into alternating electron dense and electron transparent areas, with an outer layer of loosely-associated capsular polysaccharide (Figure 16B). The cyst coat seems separated from the cell wall of the central body by 60 h of growth (Figure 17A); however, whether this is an actual event in morphogenesis resulting in the formation of a hydrated space similar to the periplasmic space between cell wall and membrane, or whether this is an artifact remains to be determined. Vesicles seem to be clustered into the electron transparent bands of the cyst coat to form a cortex-like region, surrounded on both sides with densely compressed fibrous bands. Figure 17B is representative of the 72 h sample. No further changes were evident throughout the sixth day of encystment except for the progressive reduction of PHB (Figure 18). The differentiation of the cyst coat reached a maximum at 132 h and remained unchanged at 144 h. The cyst coat appeared to consist of two distinct electron transparent bands filled with vesicles and surrounded by the denser fibrous layers (Figure 19).

Occasionally a differential staining effect was noted between capsule and cultural slime (Figure 20). The capsule retained the loosely-associated structure seen in vegetative cells, but the slime from 6 day fermentor cultures possessed a dense crystalline appearance, staining darkly with ruthenium red. These short dense fibers were similar to the structural materials of the cyst coat and thus, could be formed as a result of the presence of excess $CaCO_3$ in the culture medium.

Fig. 16. Morphogenesis of Azotobacter. Electron micrographs of thin sections of cells of Azotobacter vinelandii sampled at 36 h and at 48 h of growth. A. Cyst after 36 h of growth demonstrating progressive accumulation of cyst coat (c). B. Cyst after 48 h of growth demonstrating differentiation of cyst coat into electron-transparent and electron-dense structures (arrows). The markers represent 0.5 μm.



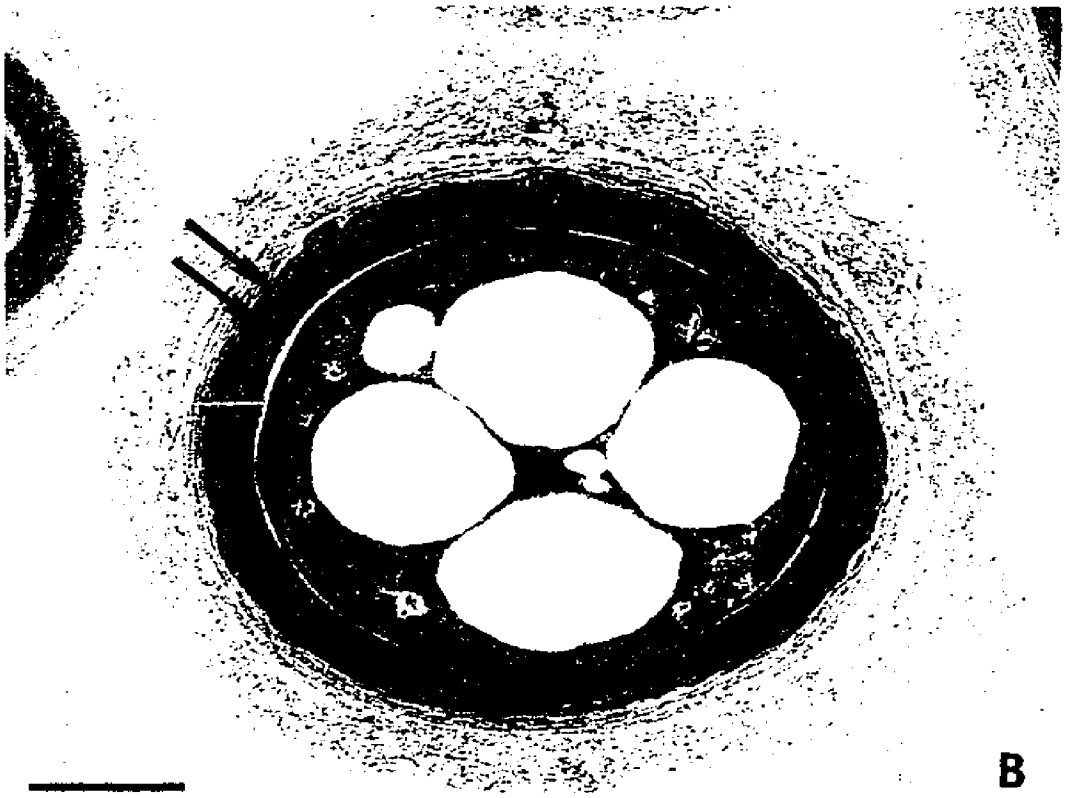
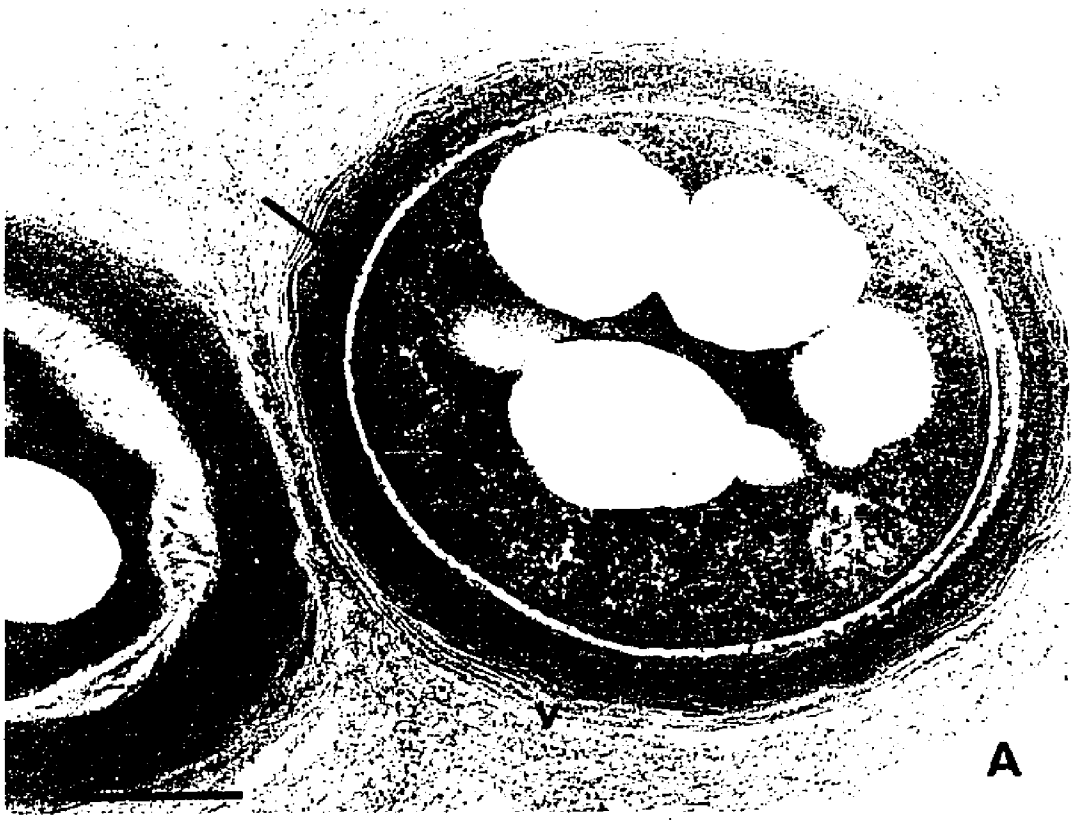

B

Fig. 17. Morphogenesis of Azotobacter. Electron micrographs of thin sections of cells of Azotobacter vinelandii sampled at 60 h and at 72 h of growth. A. Cysts at 60 h of growth with a space between the central body and the cyst coat (arrow). Vesicles (v) appear in the electron-transparent areas of the cyst coat. B. PHB granules (g) begin to decline after 72 h of growth. The markers represent 0.5 μm.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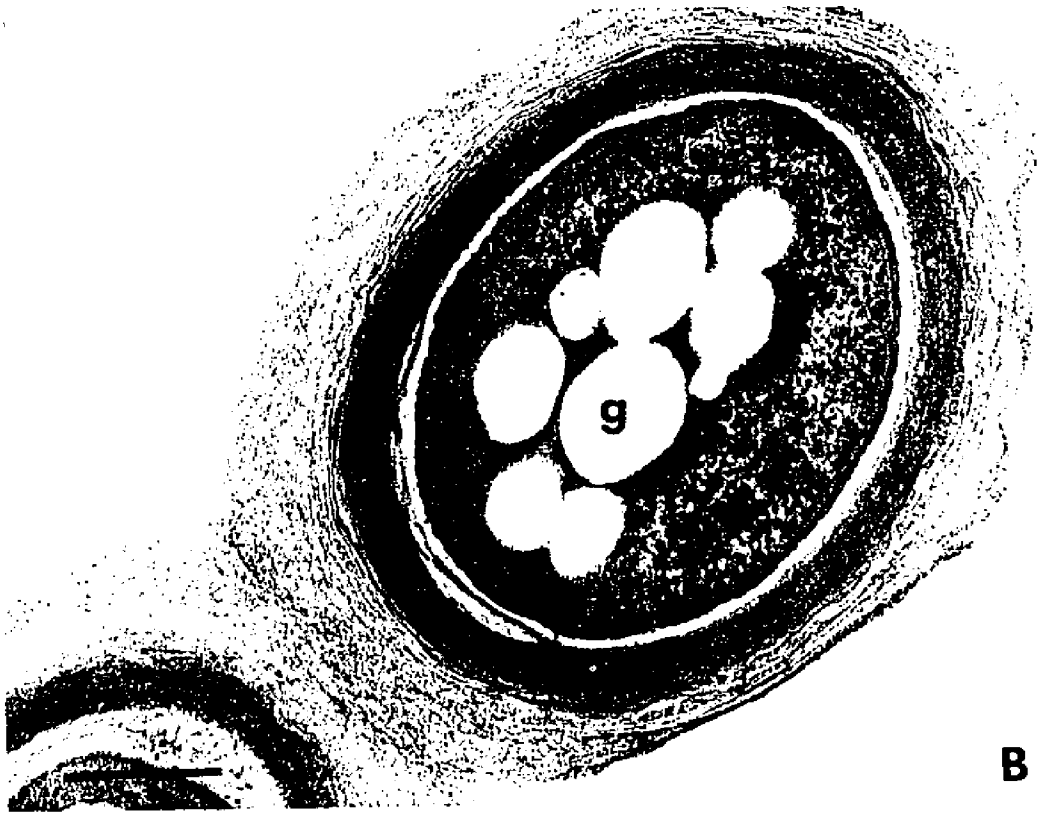
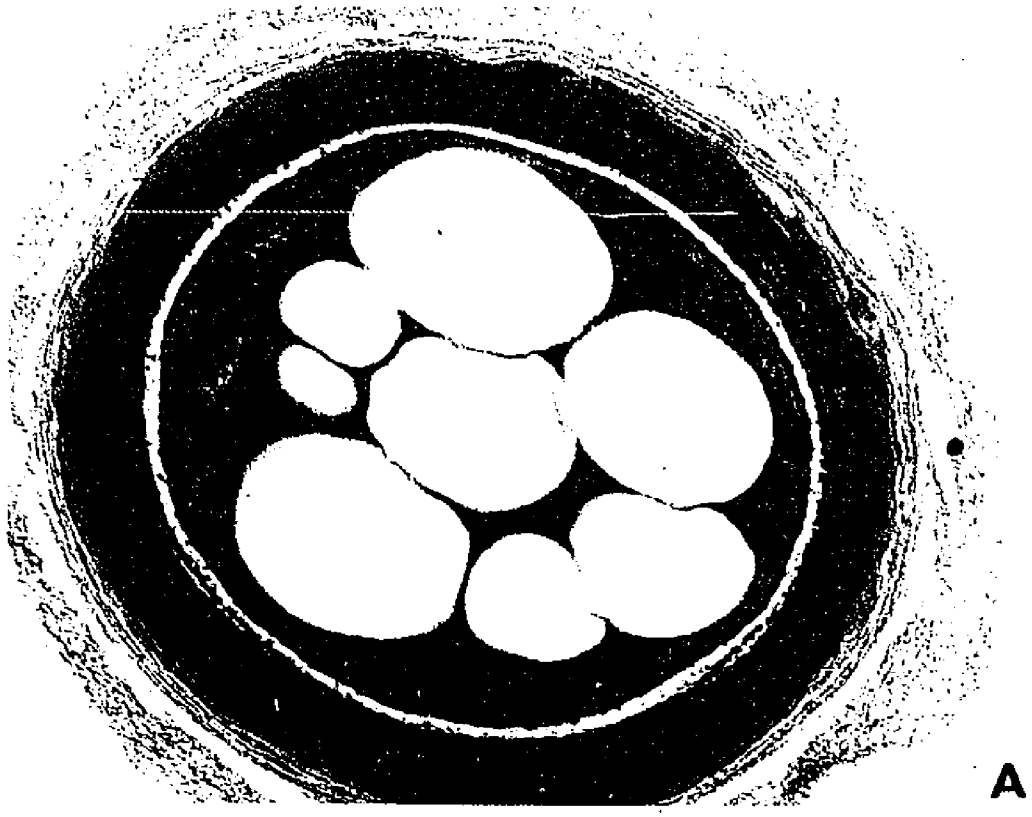

g
B

Fig. 18. Morphogenesis of Azotobacter. Electron micrographs of thin sections of cells of Azotobacter vinelandii sampled at 84 h and at 96 h of growth. A. Cysts at 84 h of growth. B. Cysts at 96 h of growth. PHB accumulations have decreased somewhat. The markers represent 0.5 μm.


A

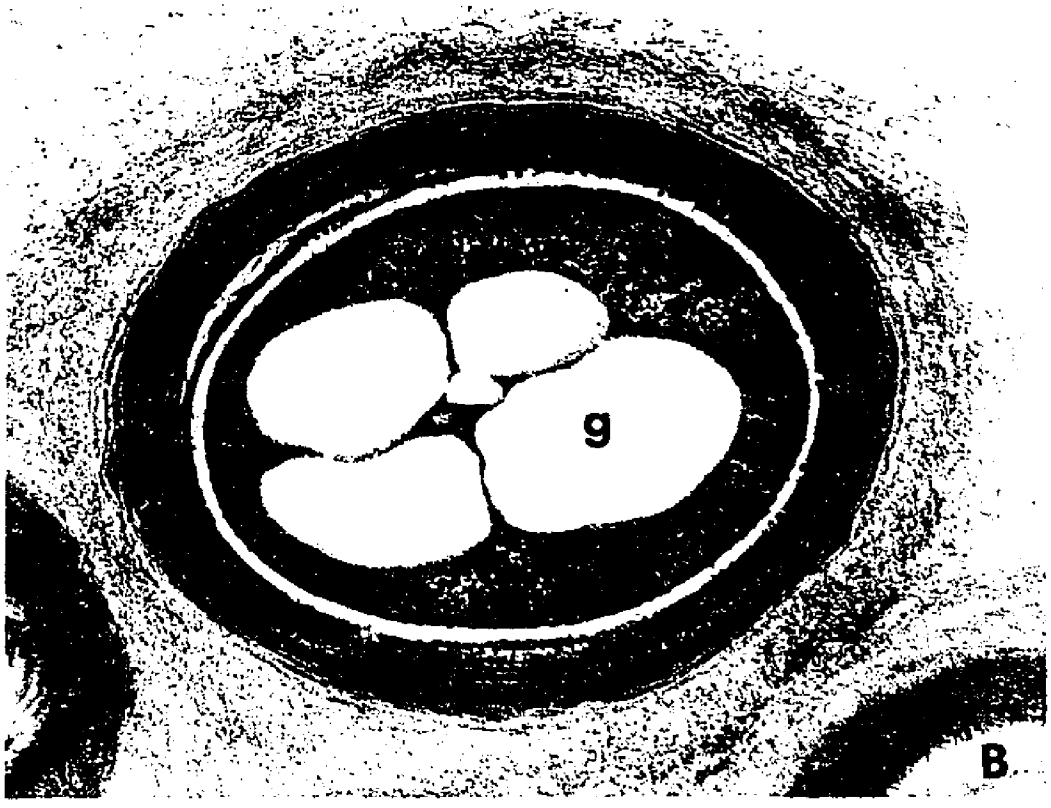
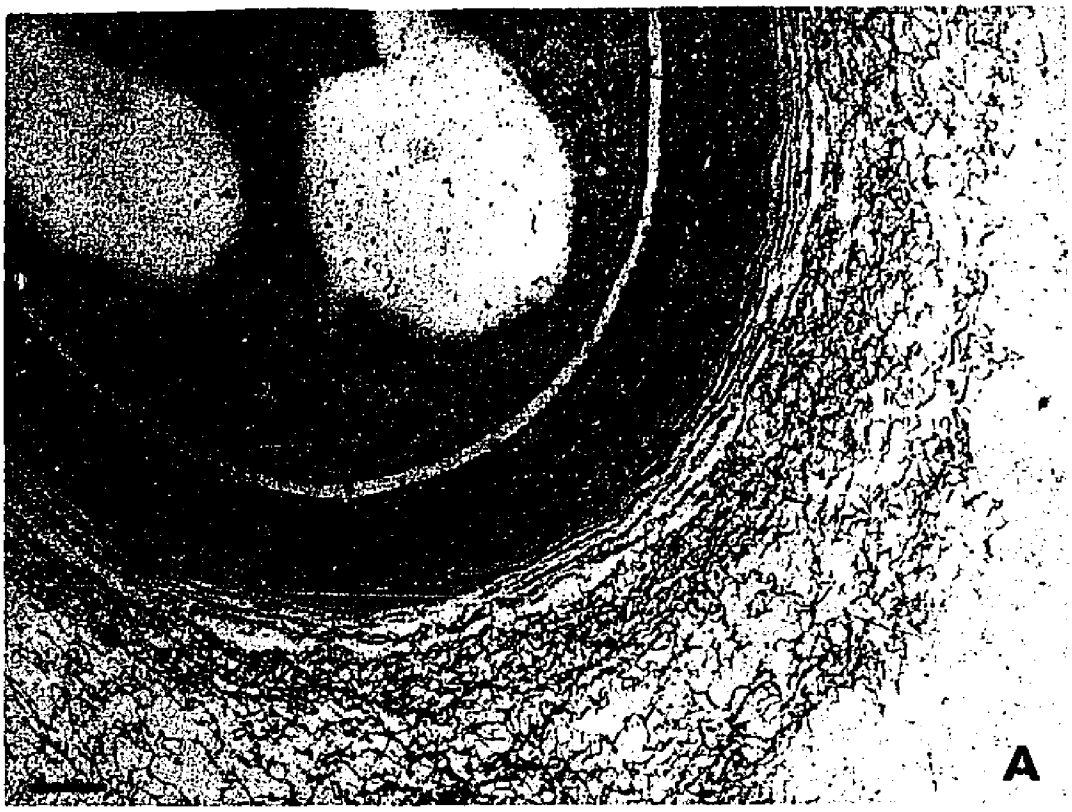

g
B

Fig. 19. Morphogenesis of Azotobacter. Electron micrographs of thin sections of cells of Azotobacter vinelandii sampled at 132 h and at 144 h of growth. A. Completion of the differentiation of the cyst coat at 132 h. B. Cyst coat is composed of alternating layers of electron-dense and electron-transparent layers (arrows). Markers represent 0.1 μm.


A

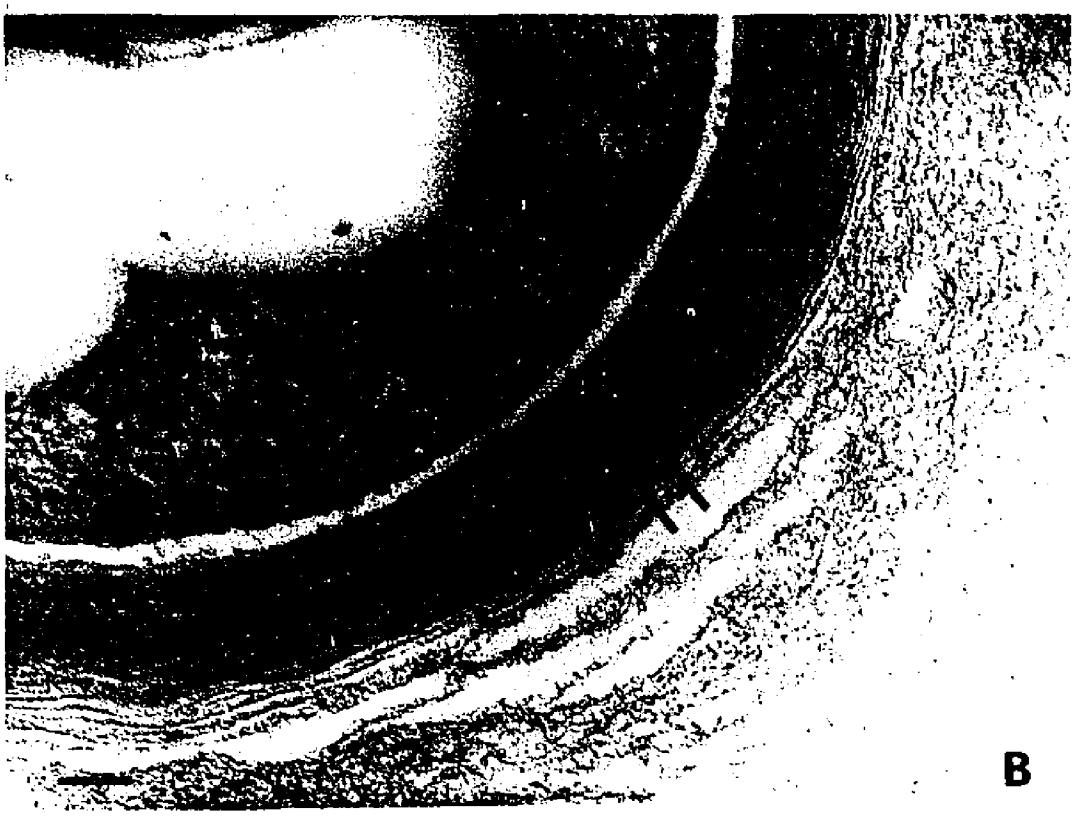
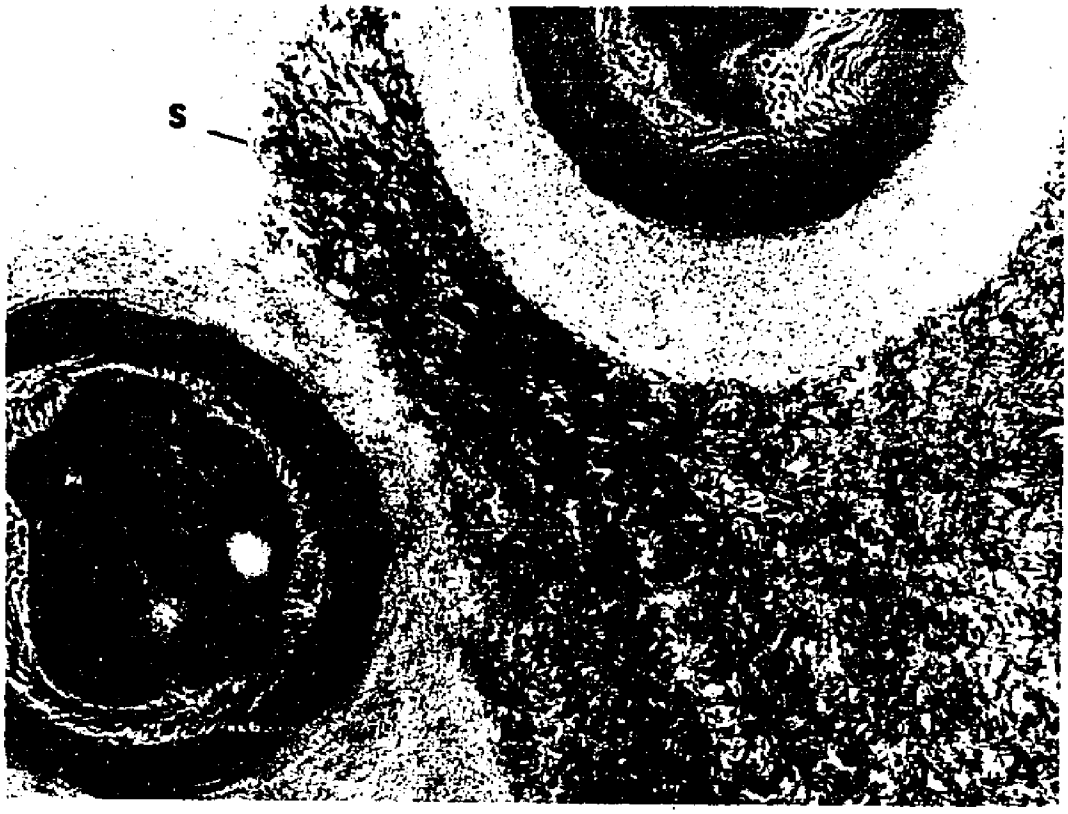
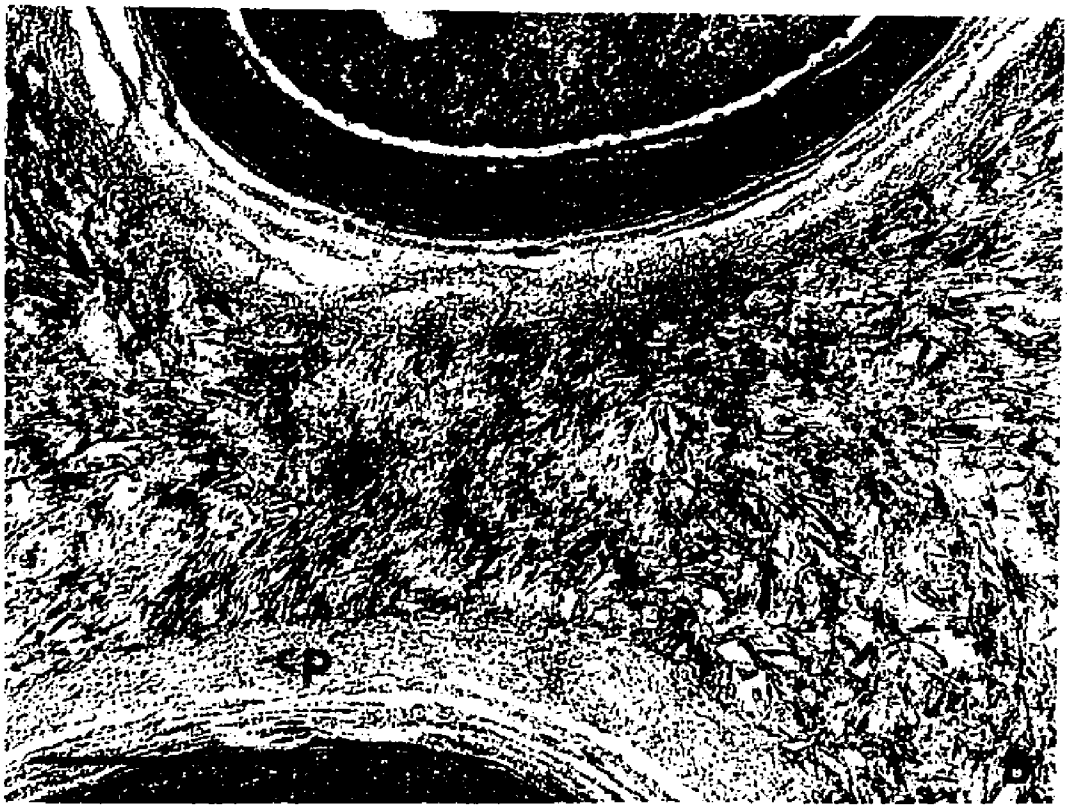

B

Fig. 20. Electron micrographs of thin sections showing the amorphous slime and discrete capsule produced by Azotobacter vinelandii after 144 h of growth in an encysting medium. A. Slime (s) stained intensely with ruthenium red. B. Capsular polysaccharide (cp) has retained original appearance and is differentiated from the amorphous slime matrix. The markers represent 0.5 μm.


S


P

Fractionation of cyst coat components

The cysts from fermentor cultures were collected utilizing a Sharples continuous flow centrifuge and the cell pastes were lyophilized. Preliminary experiments indicated that the procedure for isolation of exine developed by Lin and Sadoff (1969a) resulted in poor separation of central bodies from the crude exine. A modification of this procedure is presented in Figure 21. Exine could be freed of most of the remaining central bodies by resuspending pellet P_3 and centrifuging this suspension at 1500 x g for 15 min. The exine, remaining in the supernatant S_4, was layered onto 42% sucrose discontinuous gradients for final removal of contaminating central bodies. The purified exine fraction was lyophilized and stored in a vacuum desiccator. The EDTA soluble material contained in supernatant S_3, was recovered by ethanol precipitation and stored in a vacuum desiccator.

The effect of EDTA on the cyst is presented in Figure 22A. The exine ruptured at a single point and the central body was ejected. EDTA solubilized both intine and intine vesicles. The exine remained as the bag-shaped macromolecule presented in Figure 22B. The exine fraction was completely free of central bodies or contaminating PHB granules.

The differences in the ability of Azotobacter to encyst, when produced by some of the cultural methods evaluated, suggested that structural products of the cyst coats were either not synthesized or not assembled. Attempts were made to isolate exine from cysts produced by these less efficient methods for comparative chemical

Fig. 21. Procedure for isolation of exine and intine from cysts of Azotobacter vinelandii.

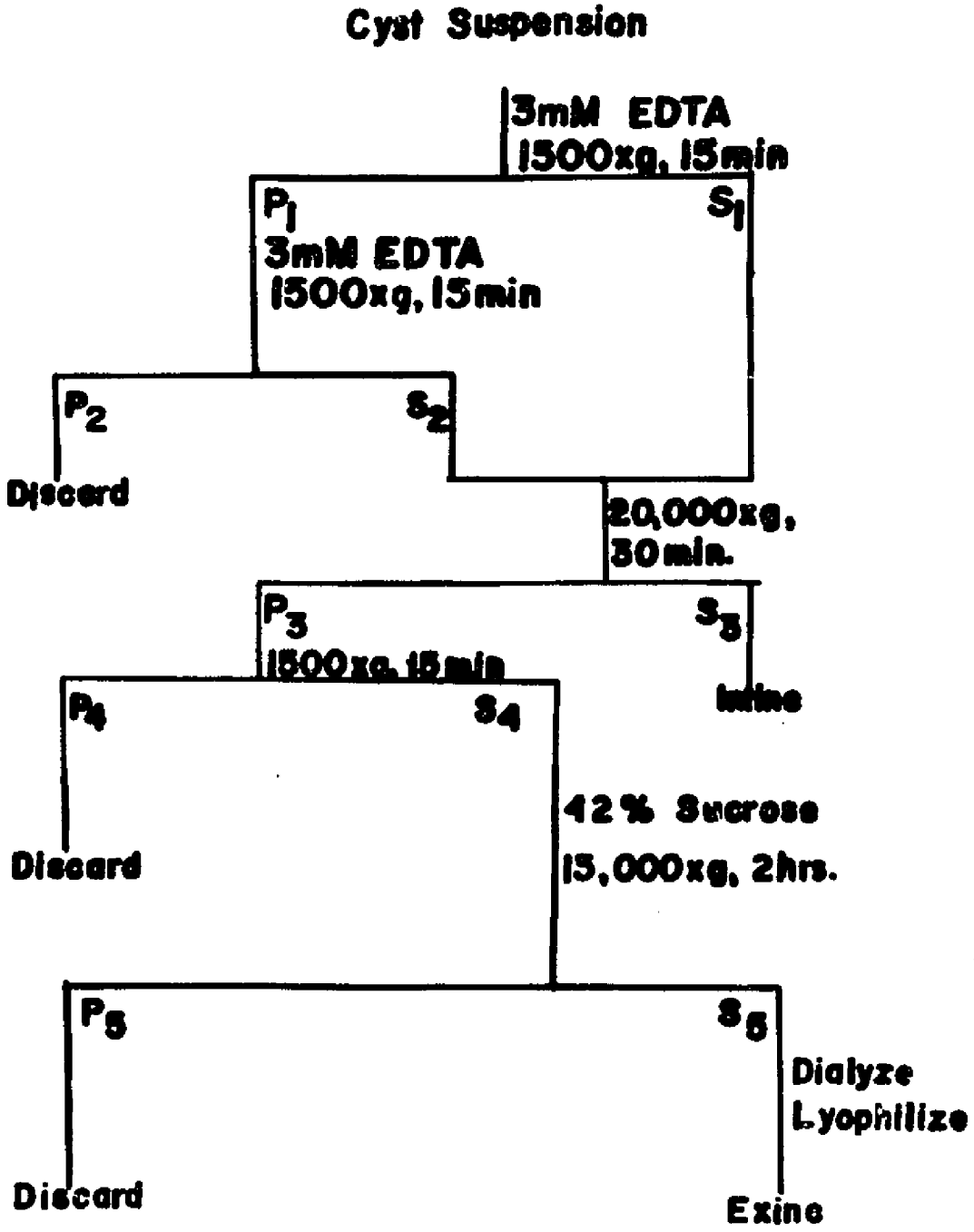
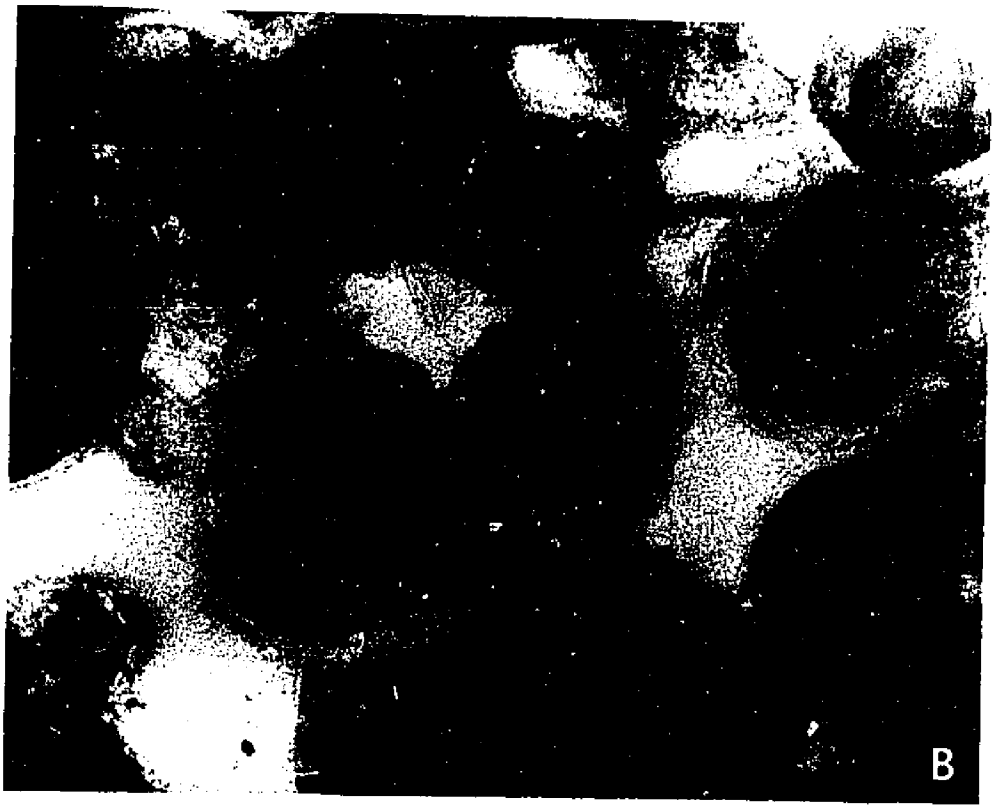

Cyst Suspension
3mM EDTA
1500xg, 15min
P1
S1
3mM EDTA
1500xg, 15min
P2
S2
Discard
20,000xg,
30 min.
P3
S3
1500xg, 15min
Intine
P4
S4
Discard
42% Sucrose
15,000xg, 2hrs.
P5
S5
Dialyze
Lyophilize
Discard
Exine

Fig. 22. Electron micrographs indicating the effects of EDTA on cysts of Azotobacter vinelandii. A. Thin section of cysts ruptured with EDTA reveals appearance of exine (e) after central body (cb) has been released. B. Negative stain of purified exine preparation. The markers represent 0.5 μm.


A


B

analysis. Treatment of cysts from these cultures with EDTA failed to effect the complete release of the central body from a large percentage of the cysts present. Additionally, the increased viscosity of cultural supernatants from BHB-supplemented cultures or cultures produced by addition of $CaCO_3$ in a dialysis bag, complicated the differential centrifugation steps of the isolation procedure (Figure 21). No attempts were made to chemically analyze these isolates since the yield of exine isolated from these cultures was low, and because the resulting exine contained relatively large amounts of capsular polysaccharides and central bodies.

The densities of the exine isolates produced by various cultural methods were determined by centrifugation in linear sucrose gradients (Table 4). These results indicate that the density of exine isolates is related to the amount of calcium present in the culture medium.

Isolation and characterization of LPS

A portion of the lyophilized cysts to be subjected to LPS extraction was resuspended in water and processed for electron microscopy. Thin sections of these control cysts are presented in Figure 23. The fully encapsulated cysts appear unaltered by lyophilization.

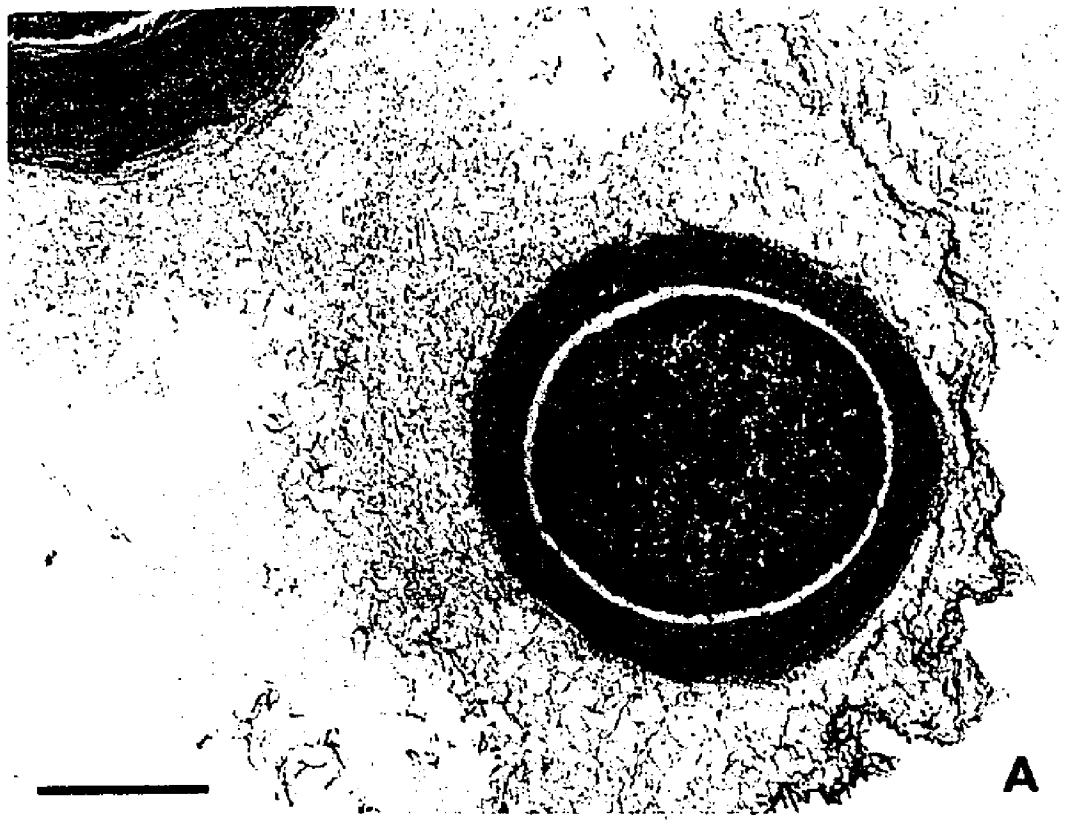
EDTA extraction

The effect of EDTA on the rupture of cyst coats and the solubilization of the vesicular component indicated that LPS might be a structural material of the cyst coat. Lyophilized cysts were extracted with EDTA according to the procedure of Leive (1965).

Table 4. Densities of exine fractions isolated from cells produced by various cultural methods.

Culture method	Density, g/cm^3
n-butanol plates	1.164
0.6% powdered $CaCO_3$	1.195
0.6% $CaCO_3$ in a dialysis bag	1.174
0.1% BHB-supplemented culture	1.170
0.1% BHB-replacement culture	1.149

Fig. 23. Electron micrographs of thin sections of lyophilized cysts of Azotobacter vinelandii prior to exposure to various extractions. A. Cysts appear relatively unaltered by lyophilization. B. Capsular polysaccharide (cp) appears intact in these lyophilized cysts. The markers represent 0.5 μm.


A


B

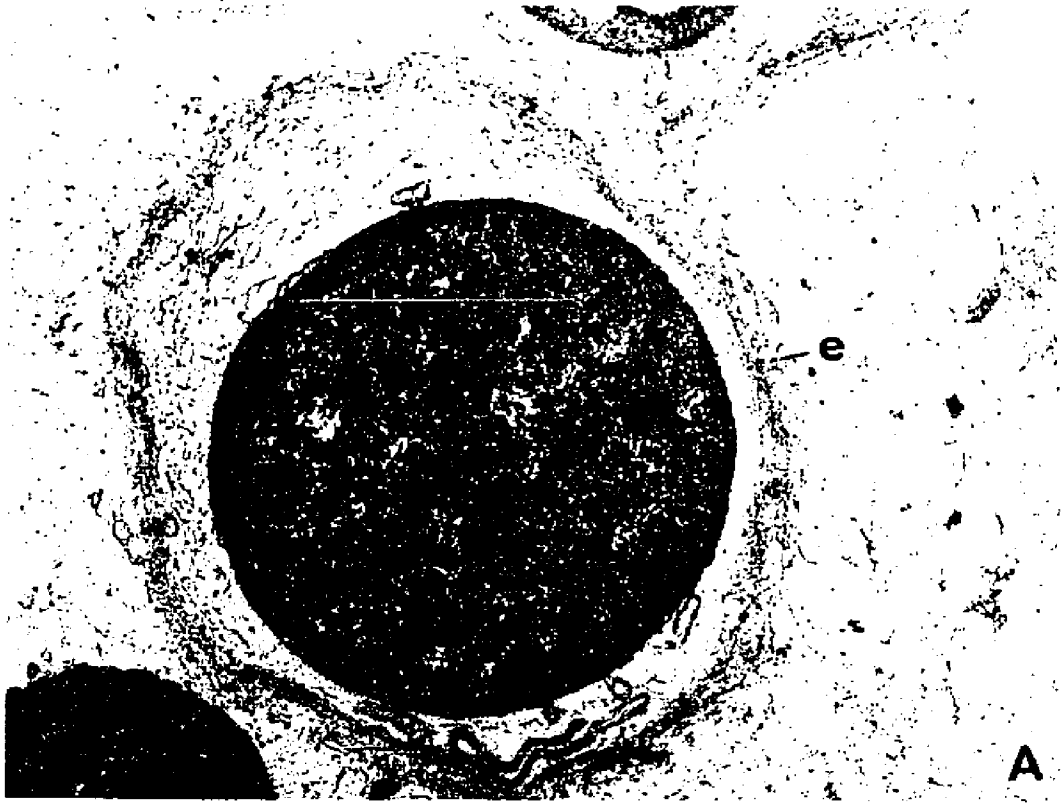
The resulting fraction was termed EDTA-LPS and consisted of a lacey, white, hygroscopic powder. Cysts were examined by electron microscopy after LPS extraction with EDTA (Figure 24). The cell wall of the central body appears intact but some vesicular outpouchings are evident. The intine is dissolved, along with the intine vesicles while the exine remains intact. Trilaminar structures thought to be residual LPS appear in the area between the central body and the exine.

Phenol extraction

LPS was also extracted from cysts by the hot aqueous phenol method of Westphal (1952). The aqueous phase containing the crude LPS was dialyzed, concentrated to 100 ml, and collected by centrifugation at 105,000 x g for 3 h. The resulting clear gel-like precipitate was dissolved in a minimal amount of distilled water and lyophilized. The cysts subjected to this extraction were prepared for electron microscopy by thin sectioning. The micrographs of phenol extracted cysts are presented in Figure 25. The outer layer of the cell wall of the central body has been stripped off. The vesicles and the electron-transparent areas of the cyst coat have been removed. Numerous trilaminar filaments, presumably LPS, appear in the area between the exine and the central body. The capsule remains undisturbed.

Since neither of these methods was successful in the quantitative removal of LPS from the cyst, a portion of cysts was exposed to the EDTA extraction for LPS, followed immediately by the phenol LPS extraction. Micrographs depicting the cysts before and after these extractions are presented in Figure 26. The cell wall of the central

Fig. 24. Electron micrographs of thin sections of cysts of Azotobacter vinelandii after extraction of LPS with EDTA. A. The central body remains virtually intact. Intine and intine vesicles are solubilized by EDTA. Ribbons of LPS (arrow) appear in the space between the exine (e) and the central body. B. The cell wall (cw) of the central body (cb) appears intact. The markers represent 0.5 μm.


e
A

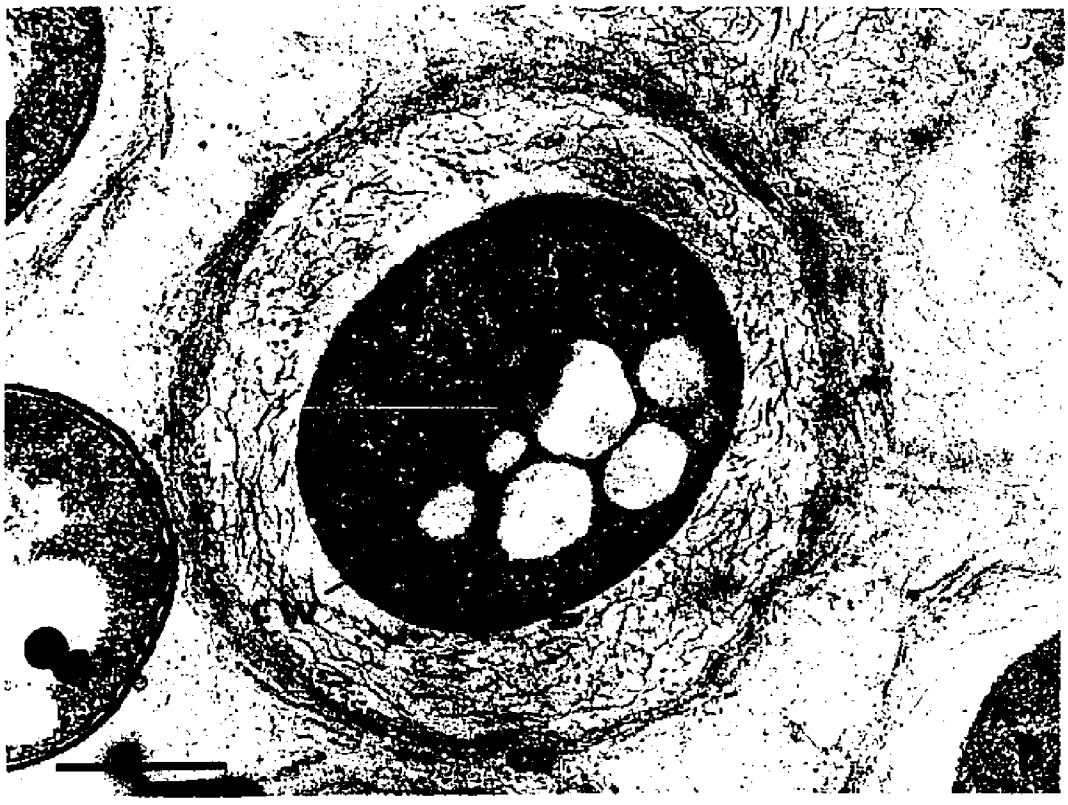
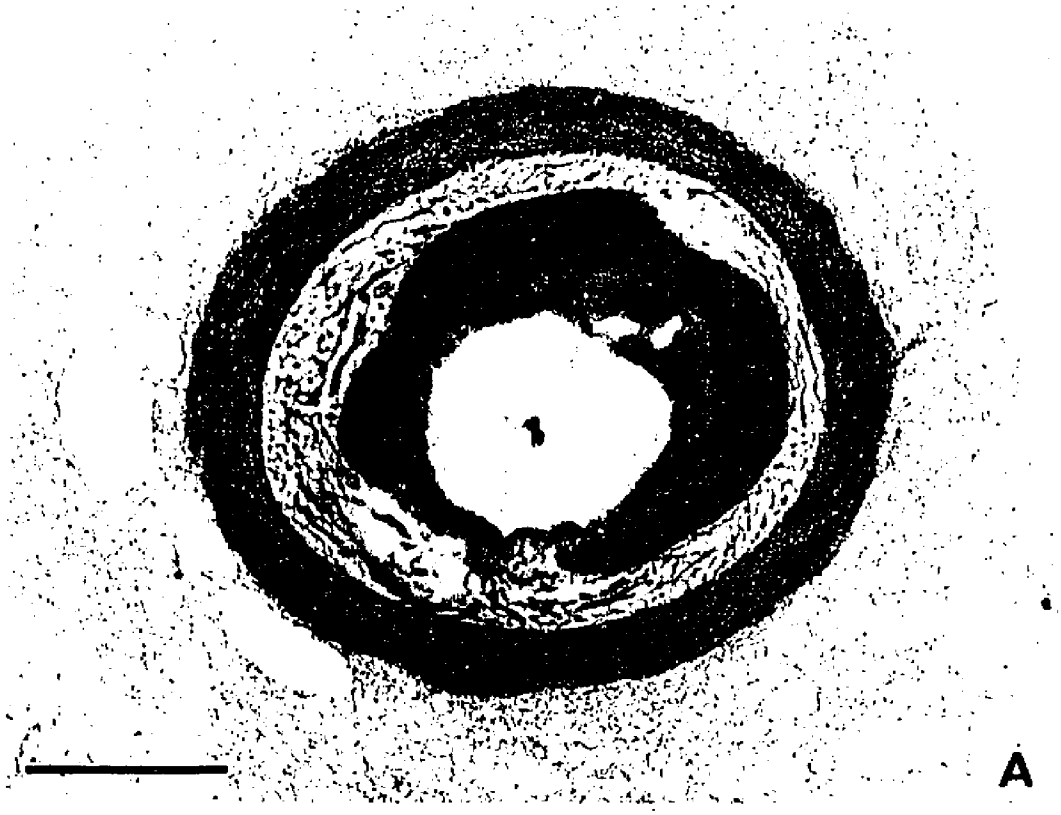
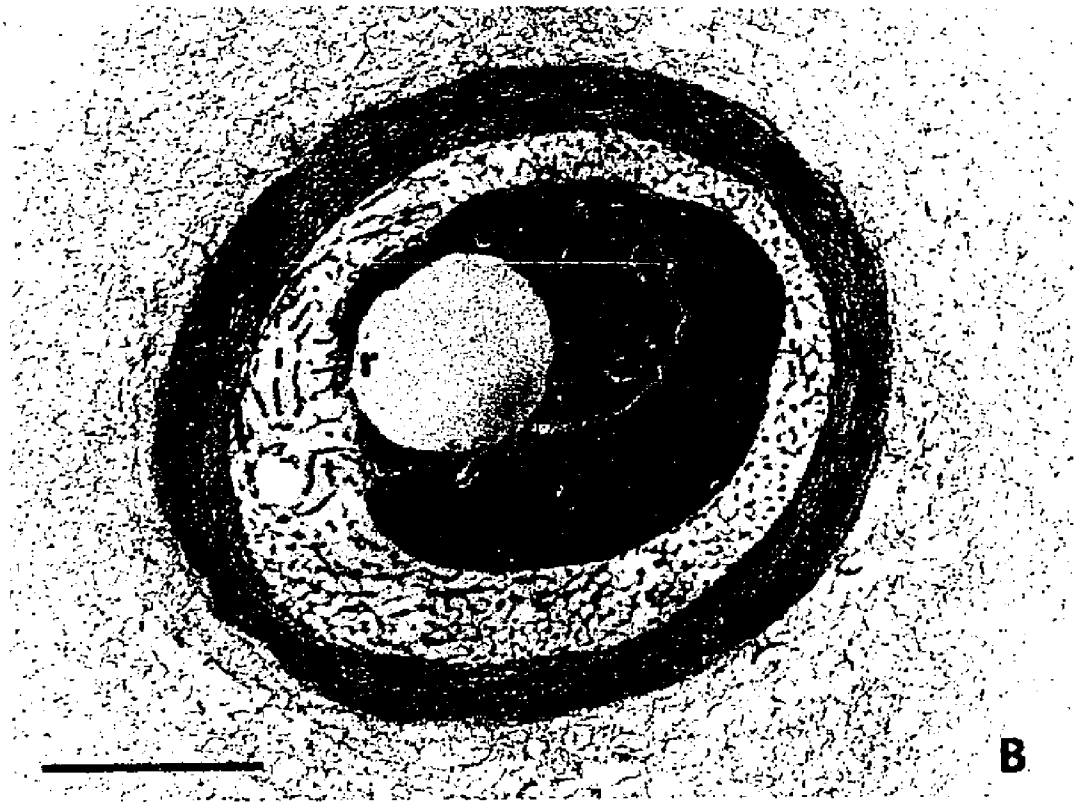


Fig. 25. Electron micrographs of thin sections of Azotobacter vinelandii after extraction of LPS with aqueous phenol. A. The cell wall of the central body has been removed. Striations of the cyst coat are no longer apparent. B. Trilaminar ribbons (r) appear between the central body (cb) and the cyst coat (c). The markers represent 0.5 μm.


A


B

body was completely removed in some areas. Large trilaminar vesicles were evident in the area between the central body and the exine. The yield of purified LPS was not significantly greater when both extraction procedures were employed.

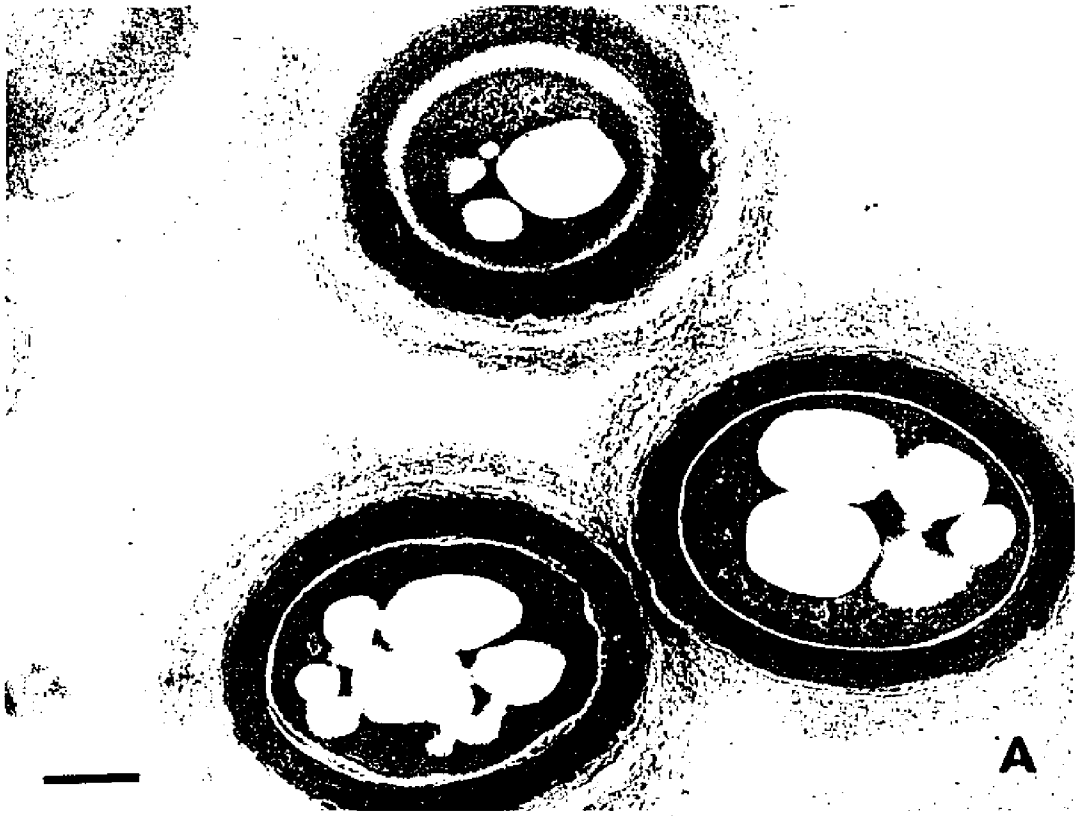
The purified EDTA-LPS and LPS fractions were dissolved at a concentration of 2 mg/ml and examined in negatively stained preparations. The EDTA-LPS appeared as a vesicular component averaging 50 nm in diameter (Figure 27A). The outer surface was trilaminar, enclosing the complex inner area. Since attempts to isolate the vesicles as a particulate fraction by high speed centrifugation failed, the formation of these vesicles is believed to be the result of spontaneous aggregation as the specimen dried on the grid. The LPS appeared as trilaminar, unbranched ribbons of varying lengths and averaging 12 nm in diameter. Occasional circular micelle-like structures were evident.

Attempts to establish the molecular weight of the LPS by glycerol gradient centrifugation were unsuccessful. The molecule exists in a polydisperse form with aggregates ranging from a molecular weight of 40,000 daltons to several million daltons.

Carbocyanin dye assay

The carbocyanin dye assay modified by Zey and Jackson (1973) was utilized for specific identification of LPS. The spectrum of the dye reagent alone and in the presence of LPS from Azotobacter vinelandii is presented in Figure 28. The dye absorbs strongly at 512 nm but undergoes a spectral shift to a maximum absorbency at 472 nm in the presence of LPS. The specificity of the shift can be

Fig. 26. Electron micrographs of thin sections of Azotobacter vinelandii before and after extraction of LPS with EDTA and phenol. A. Control cysts. B. Cysts extracted with EDTA and phenol. The cell wall of the central body has been completely removed. No differentiation appears in the cyst coat. Capsular polysaccharide (cp) remains unaltered. The markers represent 0.5 μm.


A

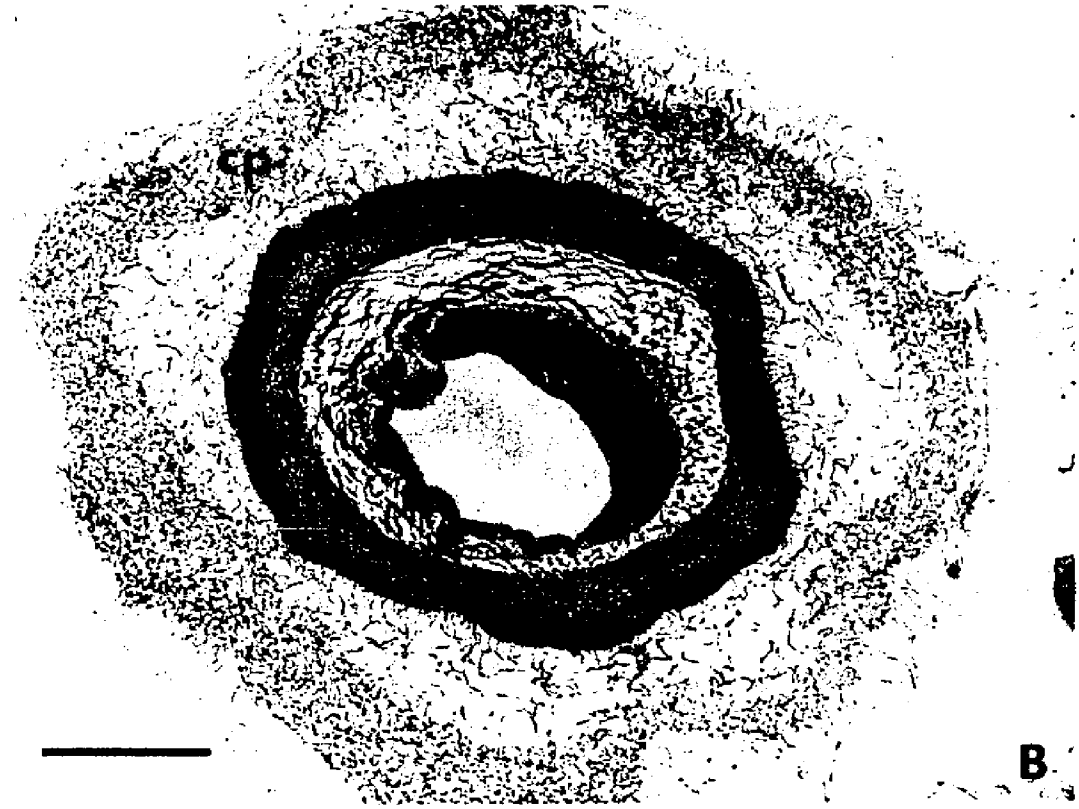
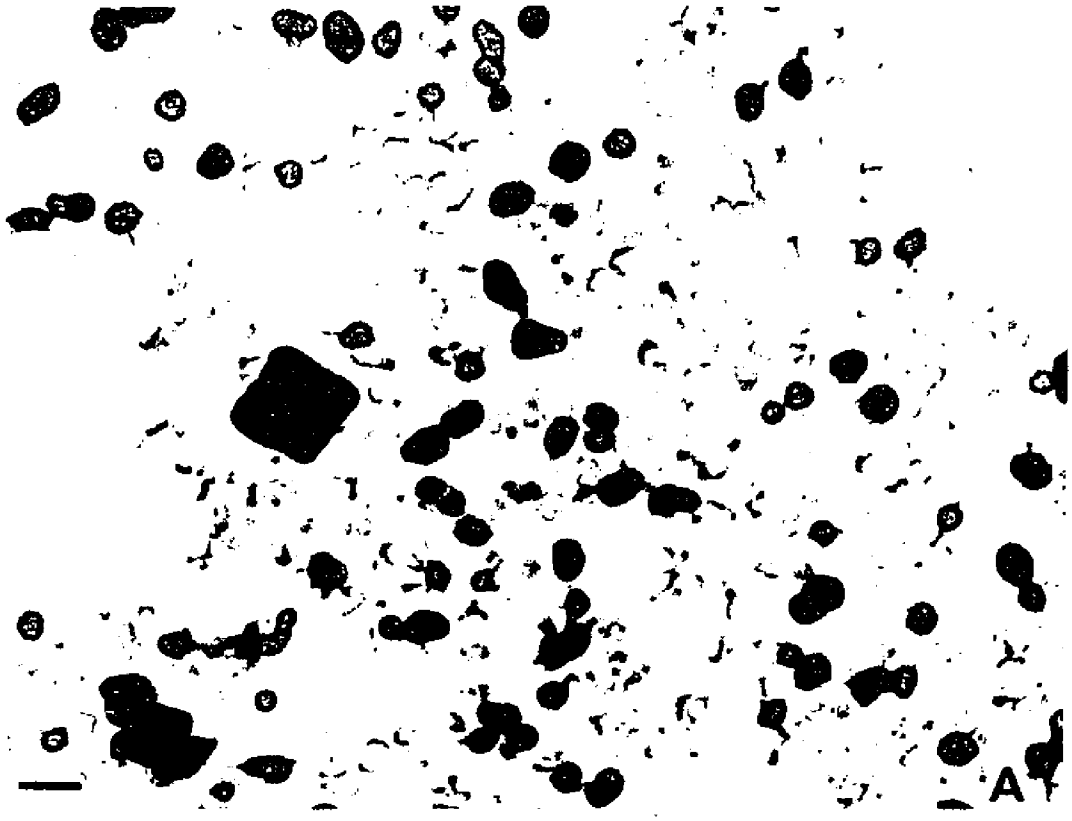

cp
B

Fig. 27. Negative stains of the EDTA-LPS and LPS fractions isolated from Azotobacter vinelandii. A. The EDTA-LPS fraction appeared as a vesicular material. B. The LPS appeared as trilaminar ribbons. The markers represent 0.1 μm.


A

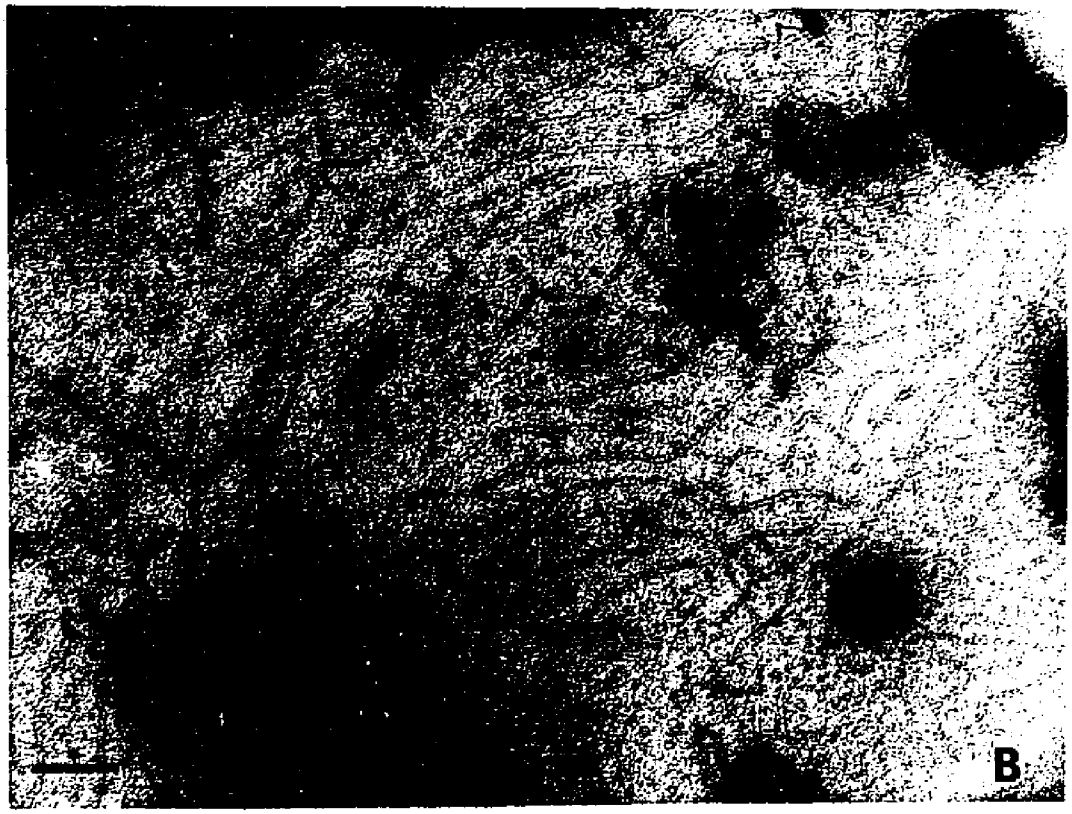
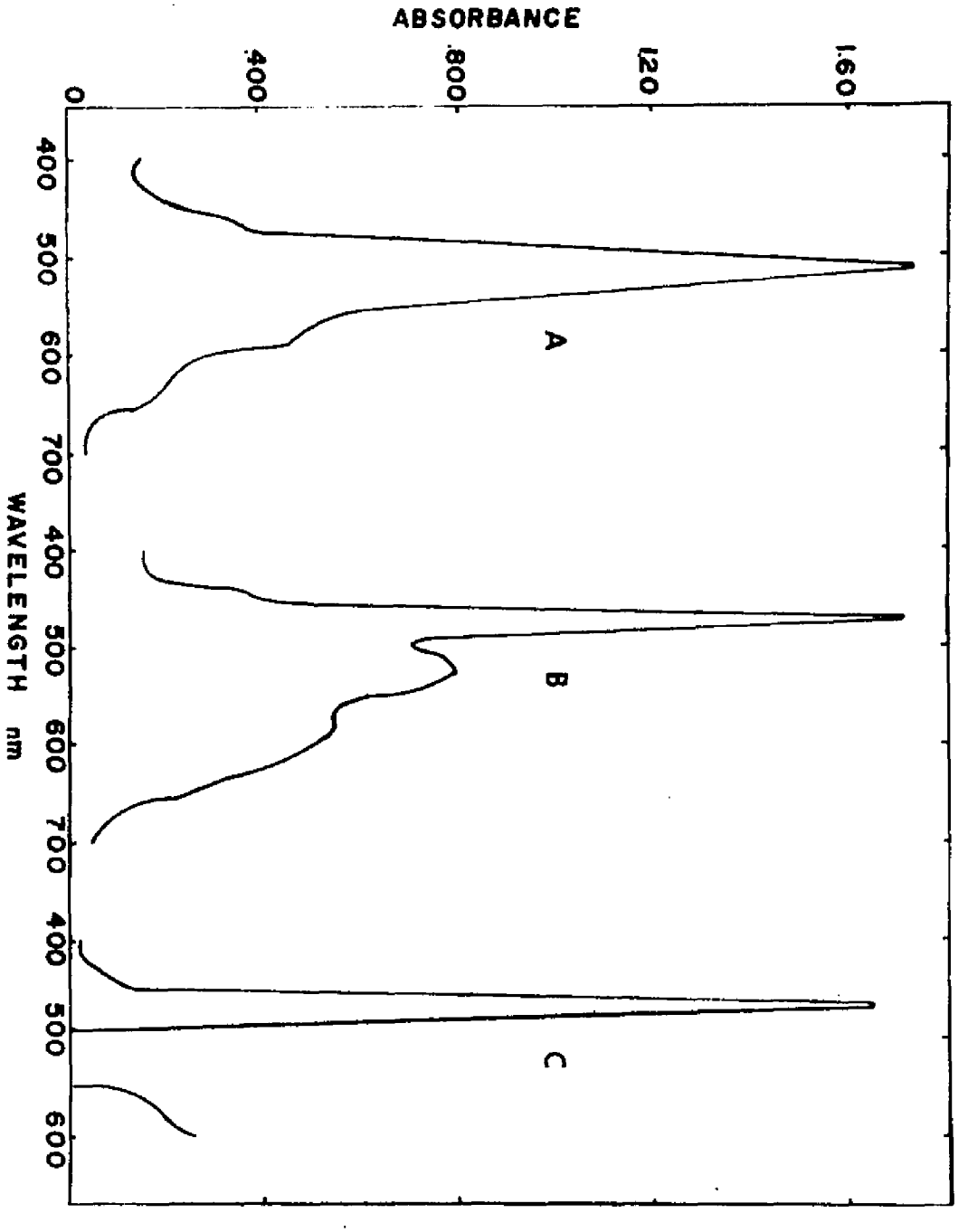

B

Fig. 28. Spectral analysis of the carbocyanin dye in the presence of LPS from Azotobacter vinelandii. A. Absorbance spectrum of the dye reagent. B. Absorbance spectrum of the dye reagent in the presence of LPS. C. Absorbance spectrum of the dye reagent in the presence of LPS when scanned against a dye blank.


ABSORBANCE
0
.400
.800
1.20
1.60
WAVELENGTH nm
400
500
600
700
400
500
600
700
400
500
600
A
B
C

enhanced by examining the LPS samples in conjunction with a dye blank to cancel residual absorbency at 512 nm. When measured amounts of LPS were reacted with the dye, a linear relationship resulted over the range of concentrations from 5 to 80 μg/ml (Figure 29). This feature enabled the quantitative determination of LPS isolated from cyst coat fractions or present in culture supernatants.

Information pertaining to the presence of LPS in supernatants of nonencysting cultures of Azotobacter is presented in Figure 30. Relatively high amounts of LPS were found in these supernatants during the first 12 h of growth. After this time, accumulation of capsular polysaccharide resulted in a spectral shift to 620 nm, characteristic of polyuronic acids (Edstrom, 1969). The presence of LPS in supernatant fluids from encysting cultures was also determined by the dye assay (Figure 31). LPS concentrations reached a maximum at 14 h of growth, after which a significant decline occurred. This decline was greater in cultures in which $CaCO_3$ had been added to the medium.

Analysis of cyst coat fractions

The identity of the saccharides contained in the exine, intine, and LPS fractions was examined in attempts to reveal information relative to the structure and origin of these cellular structures. Additionally, slime and capsule polysaccharides of vegetative cells and cysts were investigated to determine whether differences in composition could be related to the encystment process.

Fig. 29. Standard curve constructed for quantitative evaluation of LPS in culture supernatants of Azotobacter vinelandii.

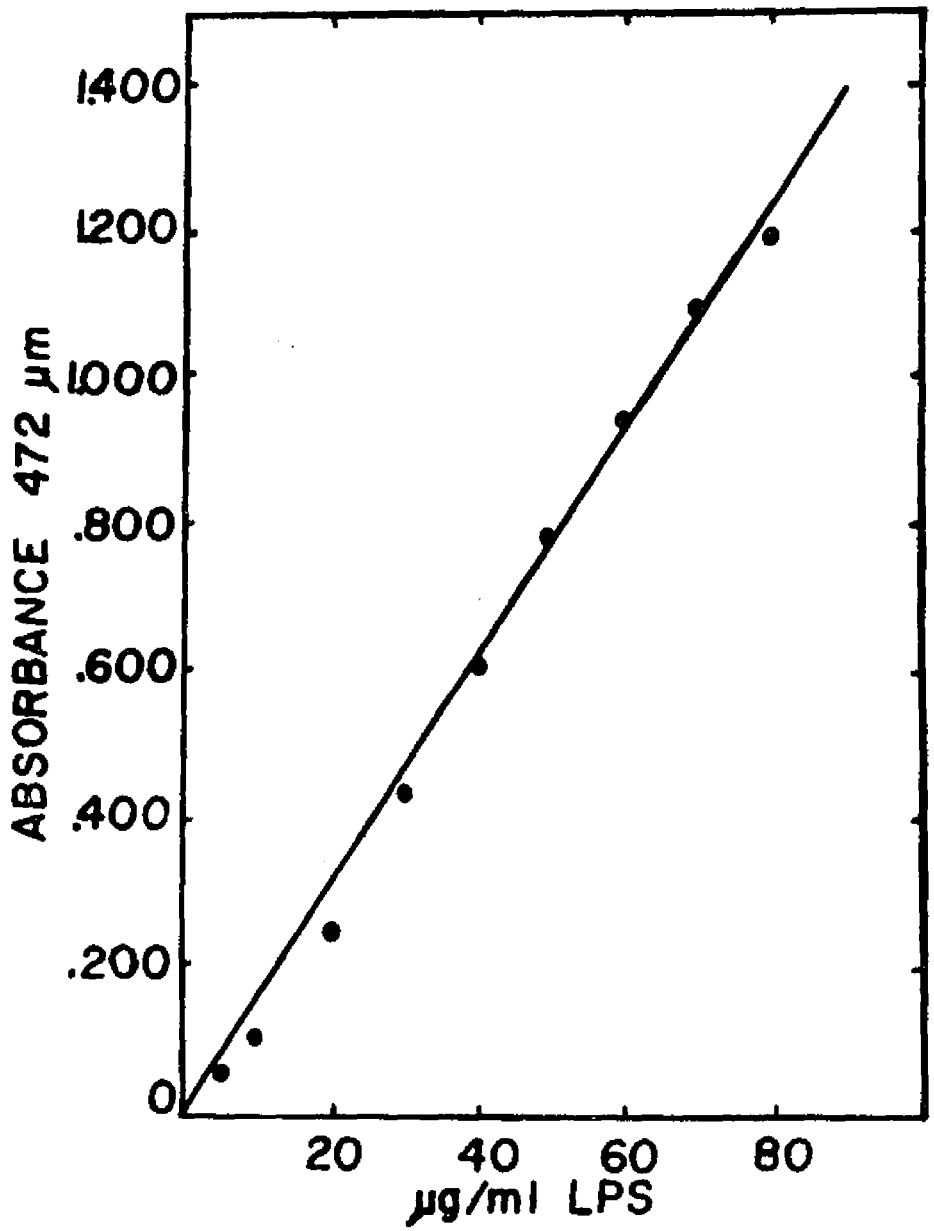

1.400
1.200
1.000
.800
.600
.400
.200
0
ABSORBANCE 472 µm
20
40
60
80
µg/ml LPS

Fig. 30. Occurrence of LPS in culture supernatants from nonencysting cells of Azotobacter vinelandii. Symbols: ●, culture buffered by the use of $CaCO_3$ in a dialysis bag; ■, control culture; ▲, BHB-supplemented culture.

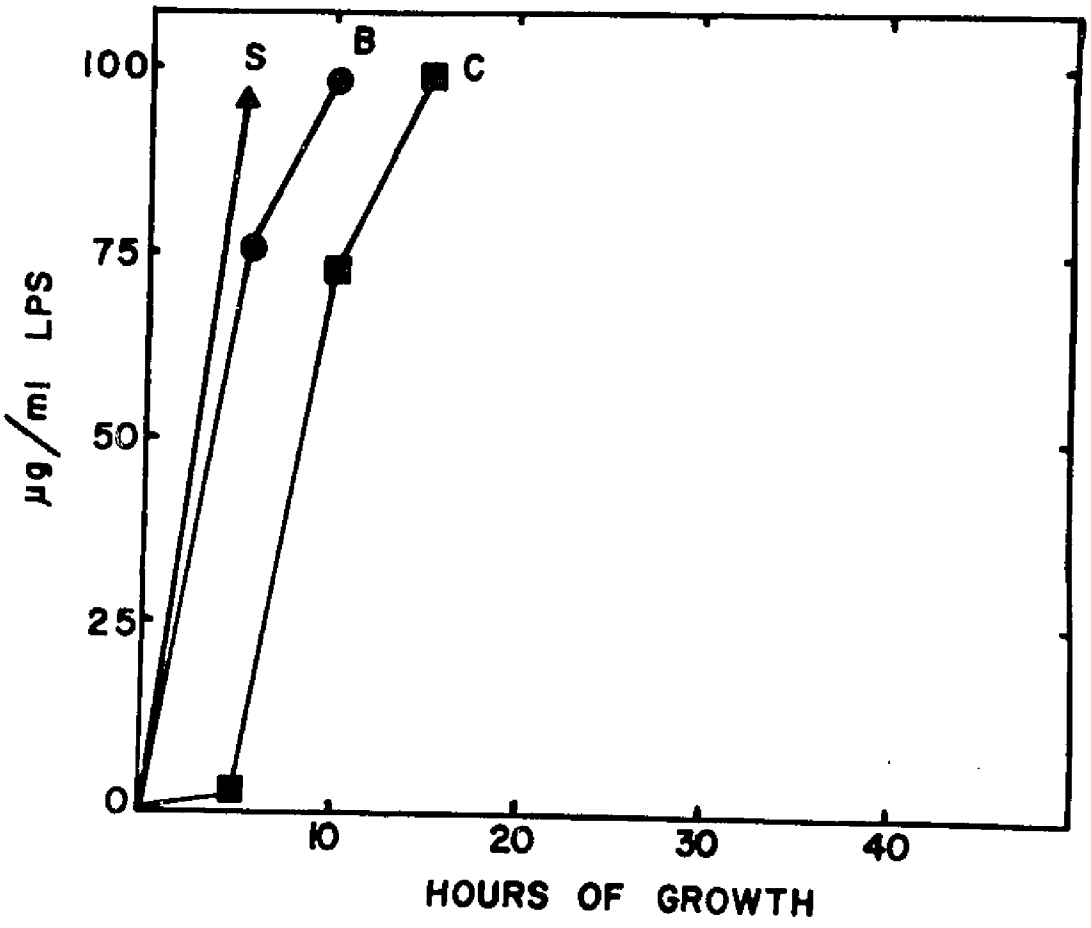
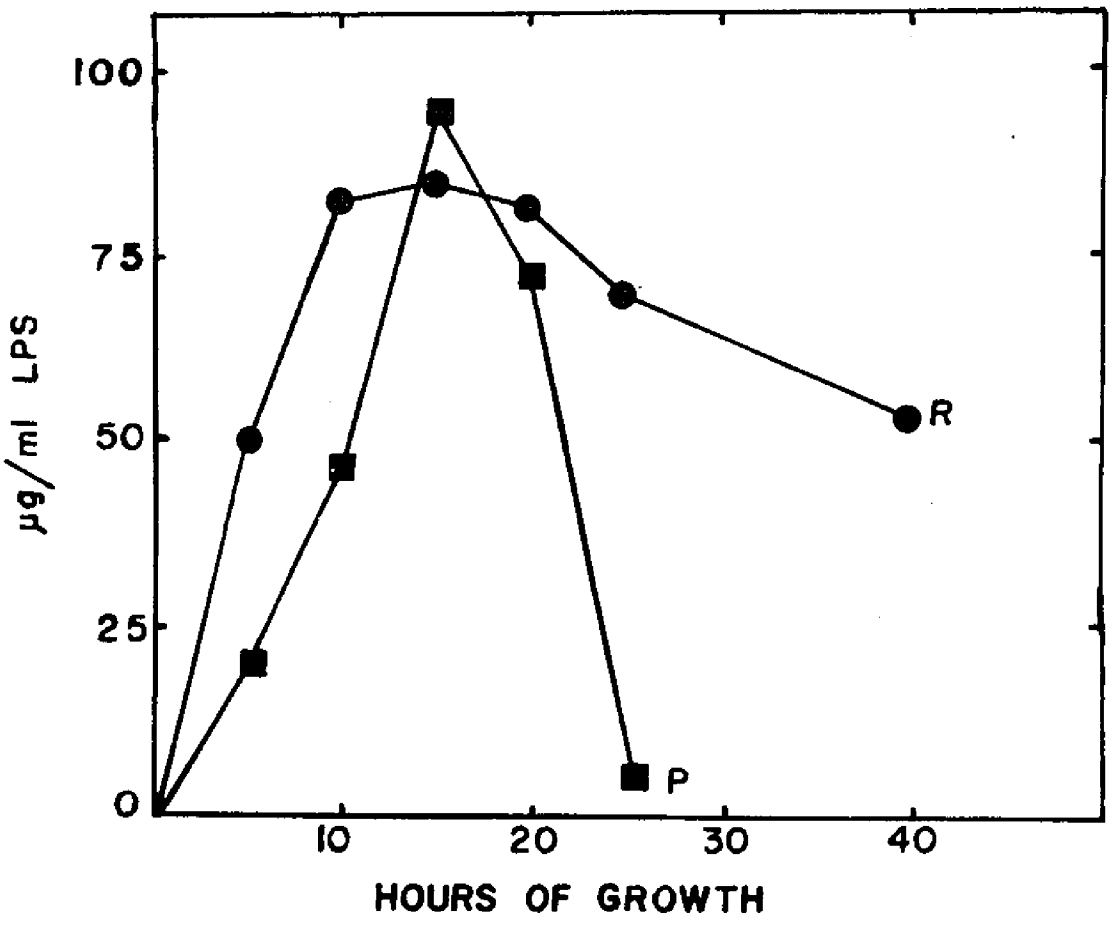

S
B
C
100
75
50
25
0
µg/ml LPS
10
20
30
40
HOURS OF GROWTH

Fig. 31. Occurrence of LPS in culture supernatants from encysting cells of Azotobacter vinelandii. Symbols: ●, BHB-replacement culture; ■, $CaCO_3$ buffered culture.


100
75
50
25
0
µg/ml LPS
10
20
30
40
HOURS OF GROWTH
R
P

Paper chromatographic analysis

In the initial phase of the investigation, the identification of saccharides in hydrolysates of various fractions was conducted utilizing ascending paper chromatography. The R_f values for the available standards are presented in Table 5. The identification of components in cyst coat fractions is presented in Table 6. Components identified in the exine fraction included glucose, galactose, glucosamine, galactosamine, and N-acetylgalactosamine. Two additional components remained unidentified. The intine material was found to contain ribose, rhamnose, mannose, glucose, and galactose. Ribose, rhamnose, glucosamine, and galactosamine were found in the LPS fraction while EDTA-LPS contained ribose, xylose, glucose, galactose, glucosamine, galactosamine, and N-acetylglucosamine. The LPS and EDTA-LPS fractions contained two additional components which remained unidentified. One of these components migrated rapidly producing a high R_f value while the other component migrated slowly, resulting in a very low R_f value. The lack of availability of additional standards for saccharides and their derivatives prevented a more complete description of the composition of the hydrolysates.

Colorimetric analysis

The partial analysis of the various cyst coat fractions by colorimetric methods is presented in Table 7. Protein, RNA, DNA, heptose, hexuronic acid and total carbohydrate analyses were performed. The results of the 2-keto-3-deoxy sugar assay are qualitative because no authentic standard was available.

Table 5. R_f values of standards utilized for paper chromatographic analysis of cyst coat fractions.

Component	R_f value		
	Solvent A	Solvent B	Solvent C
Ribose	0.519	0.658	0.372
Xylose	0.492	0.624	0.337
Fucose	0.449	0.642	0.382
Rhamnose	0.537	0.700	0.433
Mannose	0.374	0.599	0.308
Glucose	0.336	0.566	0.281
Galactose	0.298	0.514	0.253
Glucosamine	0.280	0.483	0.252
Galactosamine	0.271	0.447	0.236
N-acetylglucosamine	0.436	0.646	0.343
N-acetylgalactosamine	0.426	0.631	0.295

Solvent A. n-butanol, pyridine, water (6:4:3).
Solvent B. isopropanol, pyridine, acetic acid, water (5:5:3:1).
Solvent C. n-butanol, acetic acid, water (50:15:35).

Table 6. Saccharides and derivatives found in various cyst coat fractions by paper chromatographic analysis.

Component	Exine	Intine	LPS	EDTA-LPS
Ribose		+	+	+
Xylose				+
Fucose				
Rhamnose		+	+	
Mannose		+		
Glucose	+	+		+
Galactose	+	+		+
Glucosamine	+		+	+
Galactosamine	+		+	+
N-acetylglucosamine				+
N-acetylgalactosamine	+			

Table 7. Analysis of components in hydrolysates of cyst coat fractions by various colorimetric assay methods.

Fraction[1]	Yield, mg/g dry wt. of cysts	Percentage						
		Protein, 260/280	RNA	LPS[2]	2-keto-3-deoxy sugars[3]	Total carbohydrate	Heptose	Hexuronic acids
LPS (phenol, Mg^{++})	12.1	3.1	3	93	+	84	1.5	6
LPS (phenol, Ca^{++})	12.7	2.6	4	96	+	92	2.1	5
EDTA-LPS	41.3	6.5	10		+	25	3.9	42
LPS (EDTA, phenol)	38.6	3.0	5	97	+	96	2.1	58
Intine		0.3	28			64		50
Cell slime			2			24		8
Cell capsule			19			42		47
Cyst slime			4			16		13

1. All samples were analyzed at a concentration of 100 μg/ml. The exine fraction was not analyzed because it was particulate.
2. EDTA-LPS and intine fractions resulted in a spectral shift of the dye reagent to an absorbance maximum characteristic of hexuronic acids; therefore, the concentration of LPS could not be determined.
3. No authenic 2-keto-3-deoxy sugars were available for quantitative measurements; therefore, the presence of the specific chromagen formed in the assay was interpreted to indicate the presence of the component.

Amino acid analysis

The amino acid composition of the cyst coat fractions is presented in Table 8. The present study indicates the presence of 12 amino acids in the various cyst coat fractions, predominately aspartic acid, lysine, serine, glutamic acid, glycine, and isoleucine. Histidine, arginine, proline, and methionine were absent. The LPS contained glucosamine and galactosamine.

SDS-gel electrophoresis

Attempts to characterize the protein component of the various fractions were made utilizing SDS-gel electrophoretic techniques. Gels were stained with Coomassie blue to localize the protein component and Alcian blue to localize the carbohydrate moiety of any existing glycoproteins. These studies indicated that EDTA failed to release a significant protein component from the cell wall of the central body or from the cyst coat. This is in contrast to the large amount of protein contained in EDTA extracts from pseudomonads (Leive, et al., 1968; Rogers, et al., 1969; and Stinnett, et al., 1973).

Gas chromatographic analysis

The analysis of the available standards by gas chromatography is presented in Figure 32. Arabinose and rhamnose were poorly separated. The unidentified peaks resulted from peculiarities of the column as they appeared in all samples whether standards or unknowns.

Analysis of the slime from vegetative cells indicates a predominance of glucose with a small amount of ribose (Figure 33). The peak with a retention time of 10.5 min remains unidentified but

Table 8. Amino acid analysis of cyst coat fractions.

Amino acid or Amino Sugar	$\mu M/mg \times 10^{-3}$			
	Exine	Intine	LPS	EDTA-LPS
Lysine	3	3	1	2
Histidine	0	0.4	0	0
Aspartic acid	4	5	4	4
Threonine	1	2	4	2
Serine	5	2	30	6
Glutamic acid	3	4	24	5
Proline	0	1	0	0
Glycine	7	4	9	5
Alanine	1	5	7	3
Valine	1	3	1	2
Isoleucine	6	1	1	1
Leucine	1	3	2	2
Tyrosine	1	3	0	0.1
Phenylalanine	1	1	0.5	1
Glucosamine	0	0	208	0
Galactosamine	0	0	144	0

Fig. 32. Analysis of saccharide standards by gas chromatography.

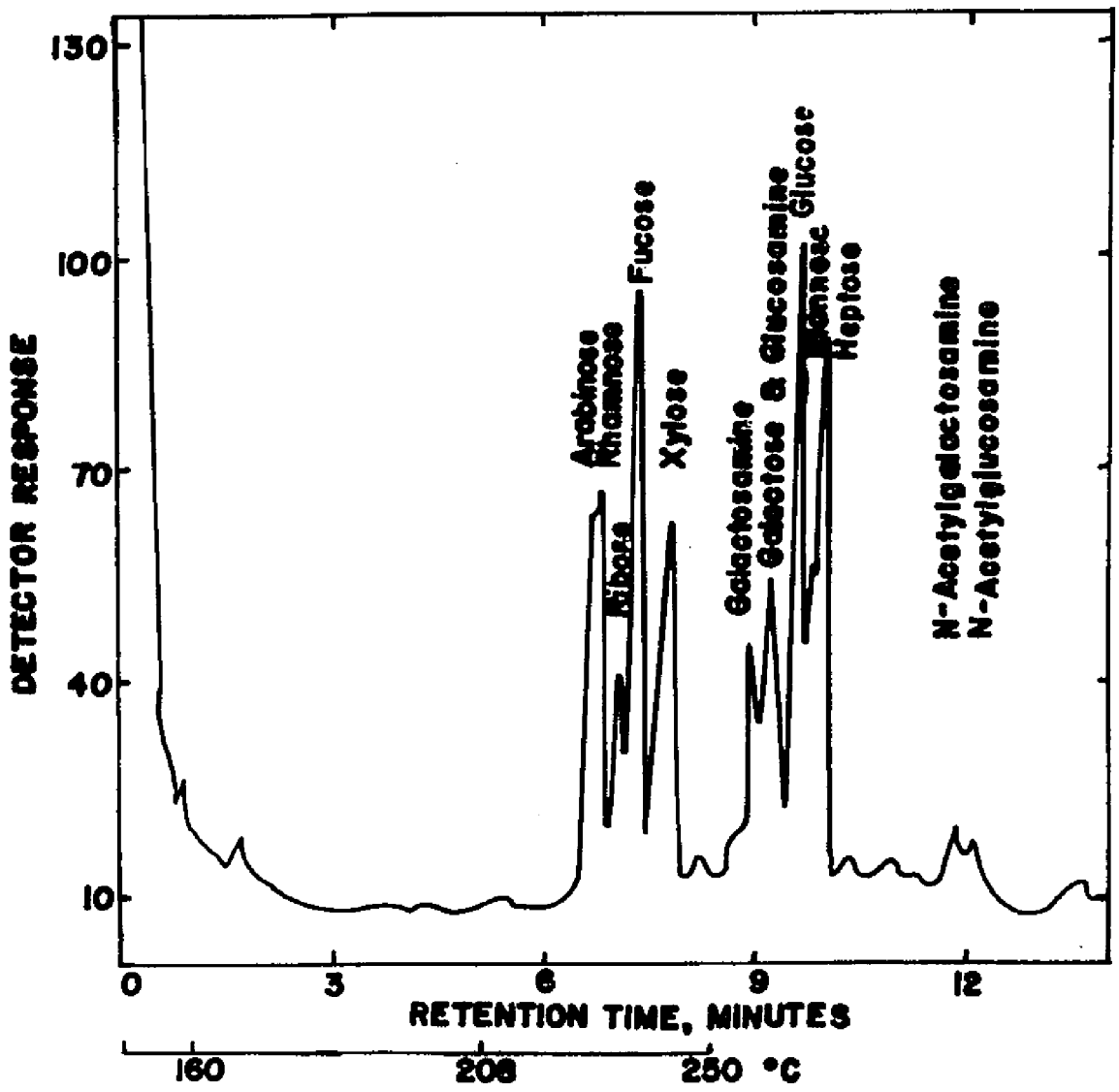
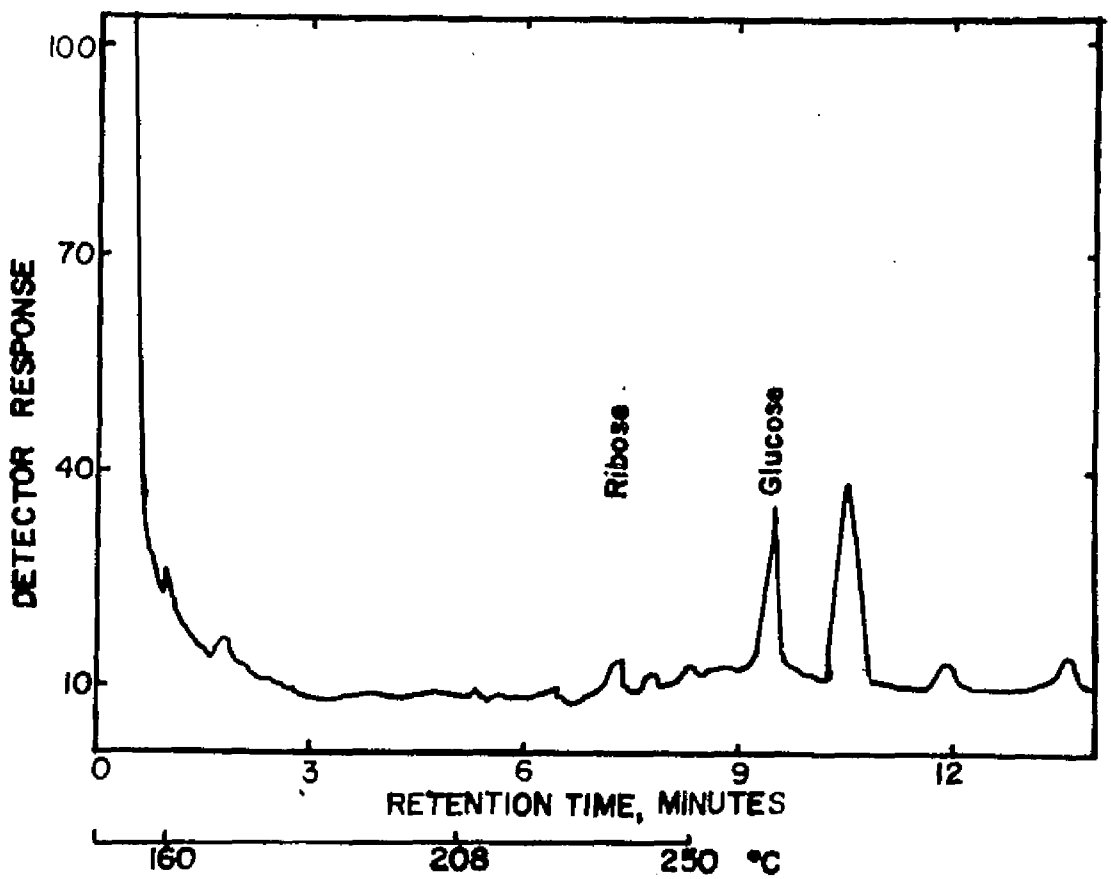

130
100
70
40
10
DETECTOR RESPONSE
Arabinose
Rhamnose
Ribose
Fucose
Xylose
Galactosamine
Galactose & Glucosamine
Glucose
Mannose
Heptose
N-Acetylgalactosamine
N-Acetylglucosamine
0
3
6
9
12
RETENTION TIME, MINUTES
160
208
250 °C

Fig. 33. Analysis of cultural slime from vegetative cells of Azotobacter vinelandii by gas chromatography.


100
70
40
10
DETECTOR RESPONSE
Ribose
Glucose
0
3
6
9
12
RETENTION TIME, MINUTES
160
208
250 °C

probably represents a uronic acid component. The capsule of vegetative cells contained glucose and two unidentified peaks, one corresponding to the suspected uronic acid peak of the cell slime, and one with a retention time of 8.8 min (Figure 34). A secondary peak at a retention time of 9.4 min existed as a shoulder upon the glucose peak but its identity also remains unknown. The composition of cyst slime is presented in Figure 35. The predominant component is glucose with lesser amounts of the unknown components found in capsule of vegetative cells. Cyst capsule could not be extracted by the method of Cohen and Johnstone (1964). Treatment of cysts with 10% NaCl adjusted to pH 9 with NaOH failed to remove capsule efficiently from cysts.

The exine contained rhamnose, ribose, galactosamine, glucose, and N-acetylgalactosamine, along with an unidentifiable component with a retention time of 10.5, probably a heptose or hexuronic acid (Figure 36).

The intine contained rhamnose, ribose, xylose, galactosamine, and N-acetylgalactosamine (Figure 37). Separation of galactose and glucosamine could not be accomplished on the column used so the peak at a retention time of 9.4 could have been either or both of these components. The large peak at a retention time of 4.5 min remains unidentified.

The EDTA-LPS fraction contained an unidentified peak at a retention time of 4.5 in addition to rhamnose, ribose, xylose, galactosamine, and glucosamine or galactose (Figure 38).

The LPS fraction contained rhamnose, ribose, and N-acetylgalactosamine (Figure 39). Although no standards were available for 2-keto-3-deoxy sugars or most of the heptoses, these peaks were

Fig. 34. Analysis of capsular polysaccharide from vegetative cells of Azotobacter vinelandii by gas chromatography.

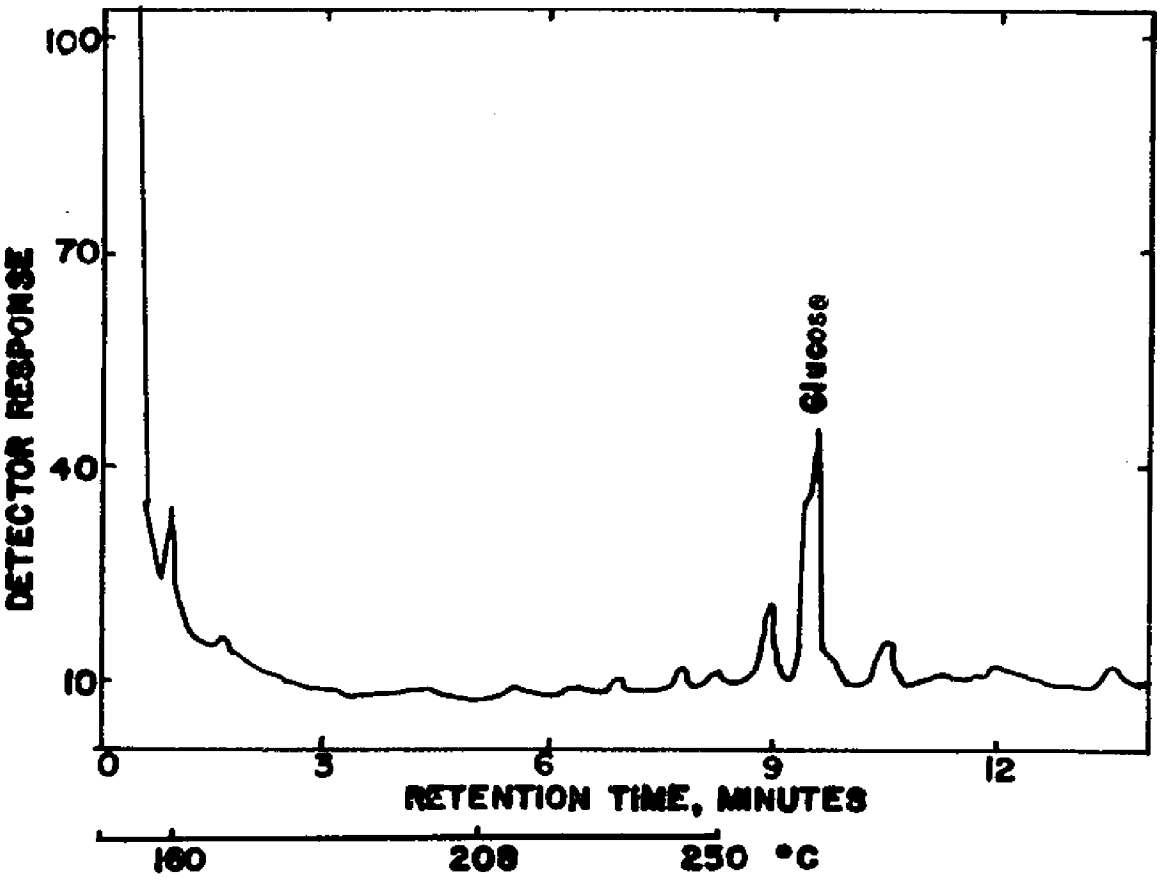

100
70
40
10
DETECTOR RESPONSE
Glucose
0
3
6
9
12
RETENTION TIME, MINUTES
160
200
250 °C

Fig. 35. Analysis of slime from cysts of Azotobacter vinelandii by gas chromatography after 4 days of growth.

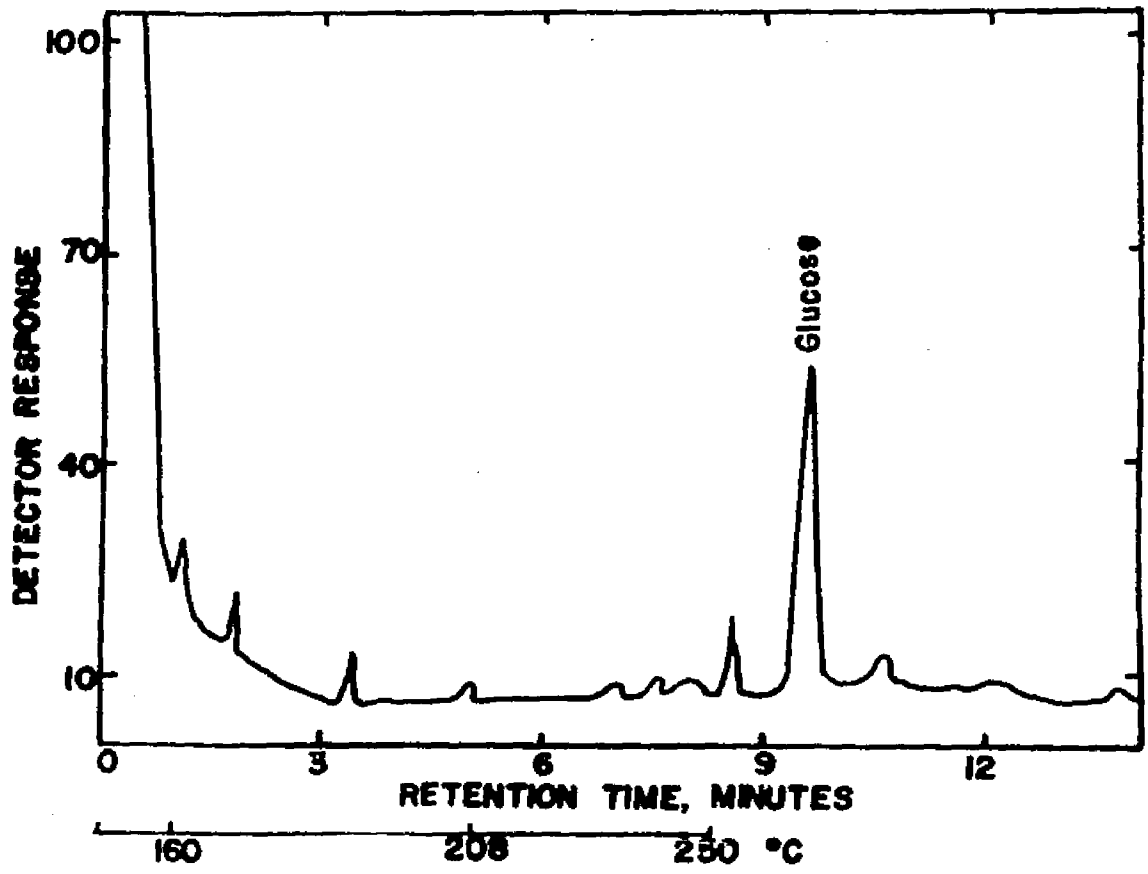

100
70
40
10
DETECTOR RESPONSE
Glucose
0
3
6
9
12
RETENTION TIME, MINUTES
160
205
250 °C

Fig. 36. Analysis of exine of Azotobacter vinelandii by gas chromatography.

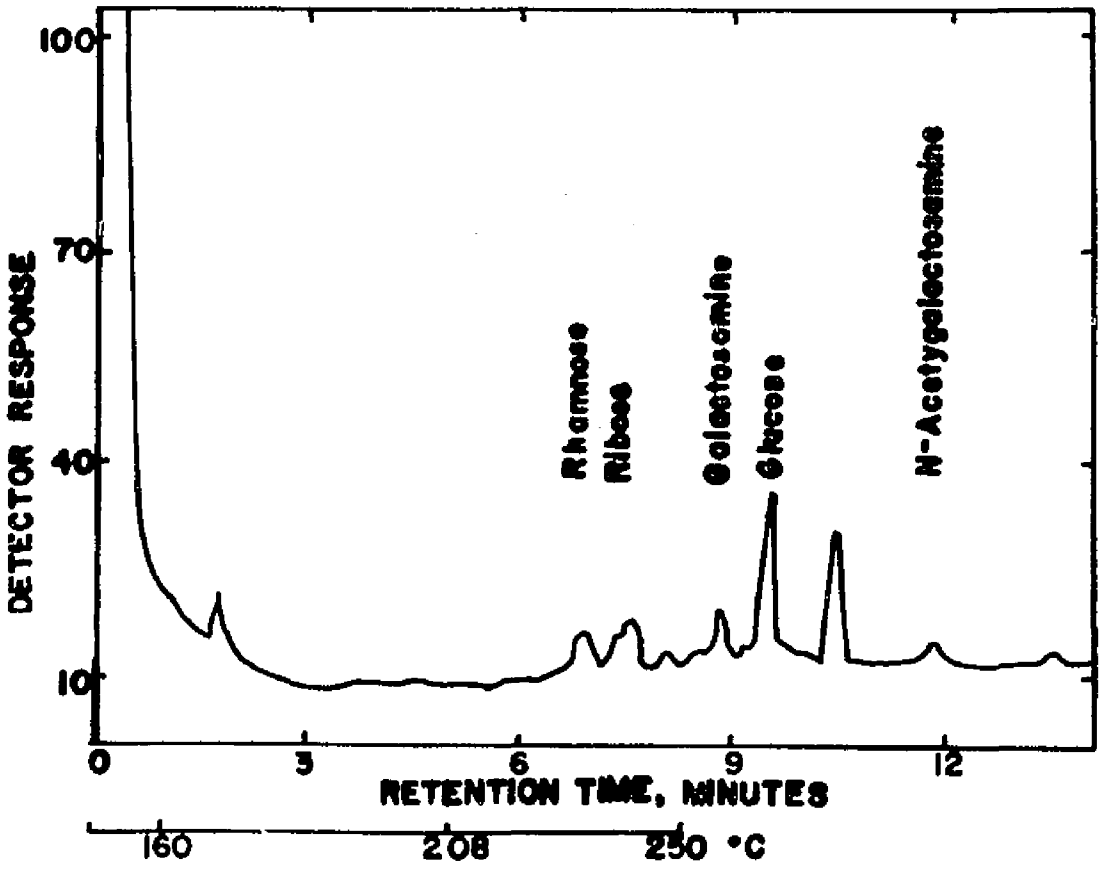

100
70
40
10
DETECTOR RESPONSE
Rhamnose
Ribose
Galactosamine
Glucose
N-Acetylgalactosamine
0
3
6
9
12
RETENTION TIME, MINUTES
160
208
250 °C

Fig. 37. Analysis of intine of Azotobacter vinelandii by gas chromatography.

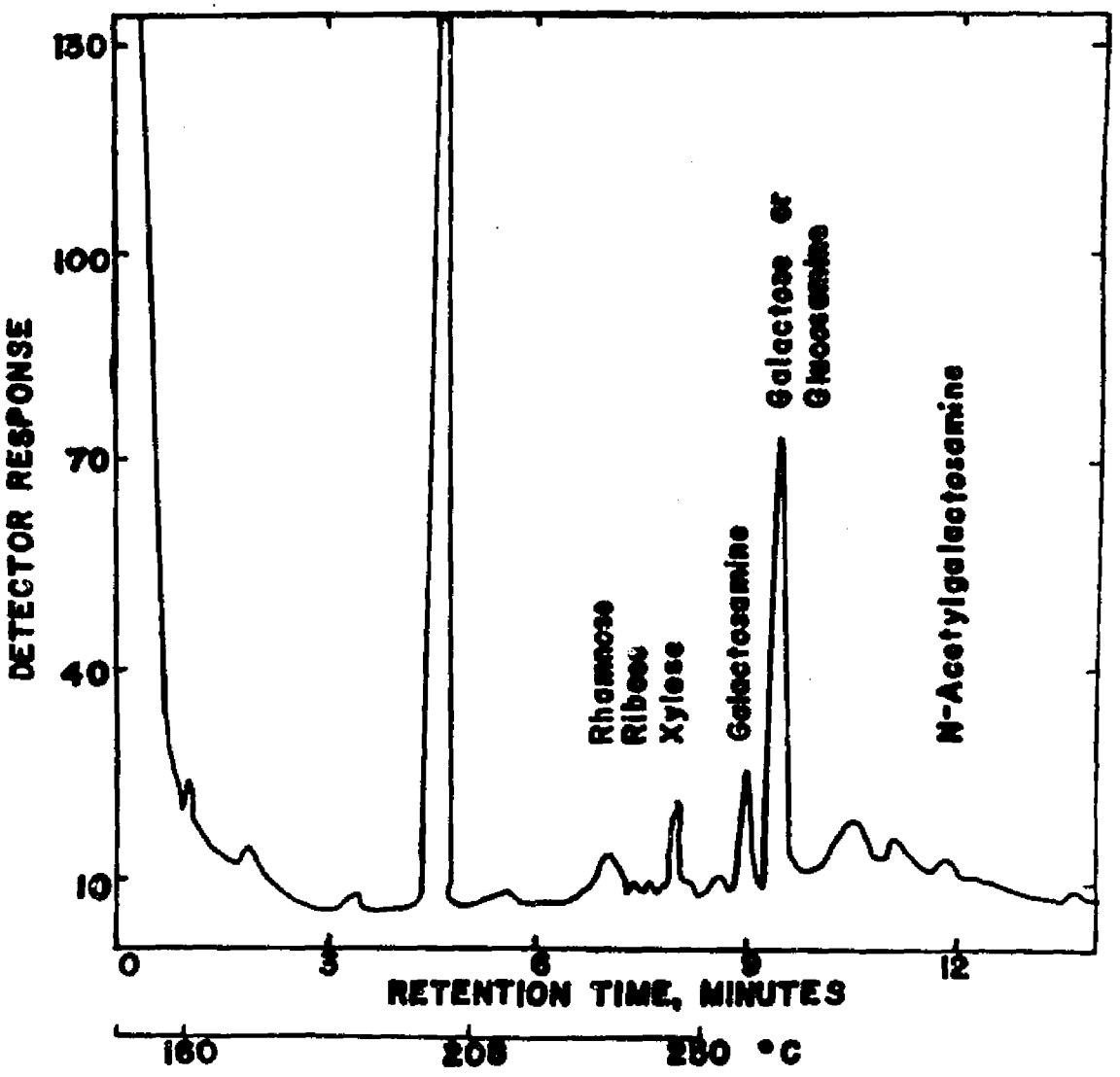

DETECTOR RESPONSE
150
100
70
40
10
Rhamnose
Ribose
Xylose
Galactosamine
Galactose or
Glucosamine
N-Acetylgalactosamine
0
3
6
9
12
RETENTION TIME, MINUTES
160
205
250 °C

Fig. 38. Analysis of EDTA-LPS material from intact cysts of Azotobacter vinelandii by gas chromatography.

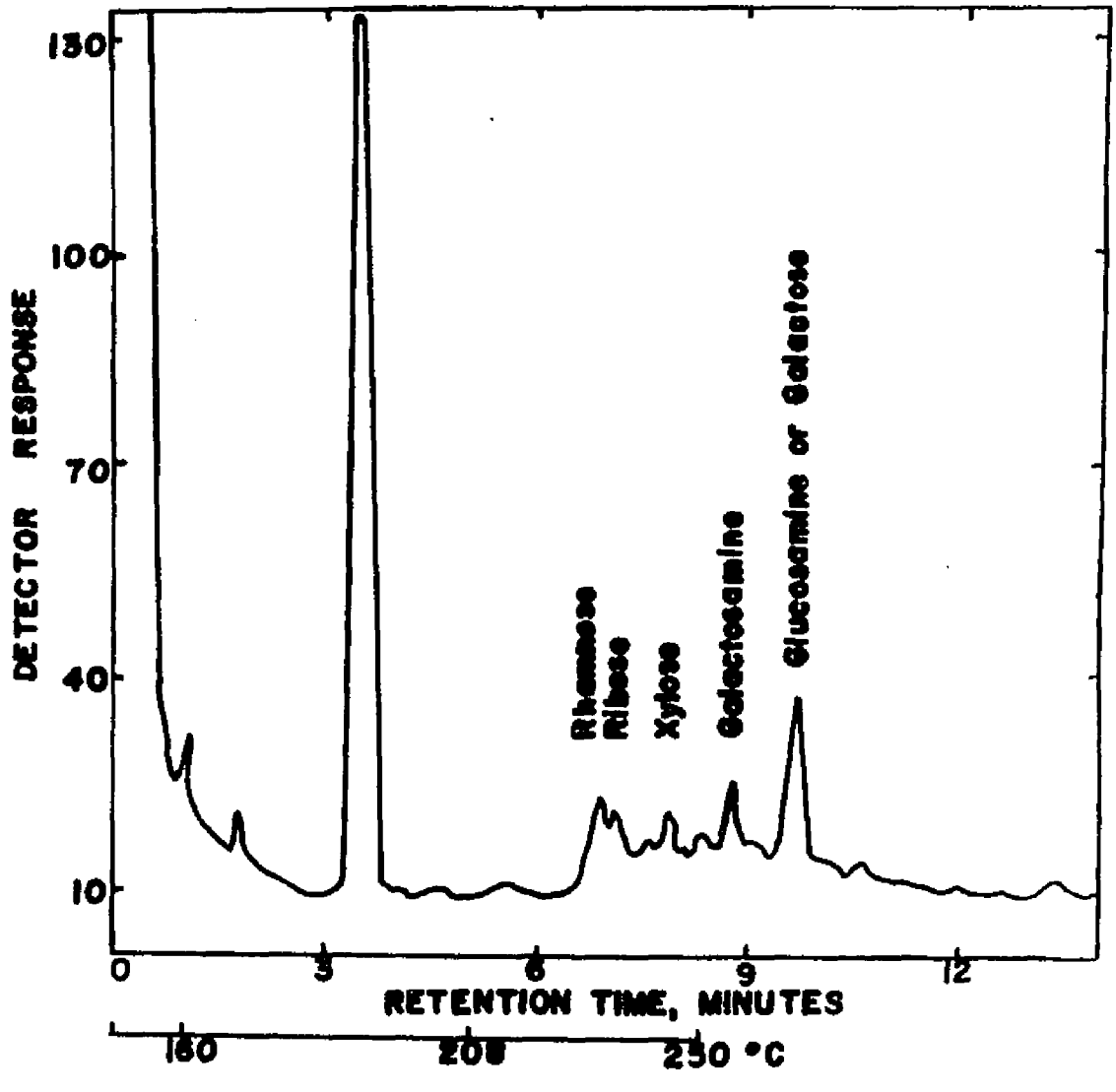
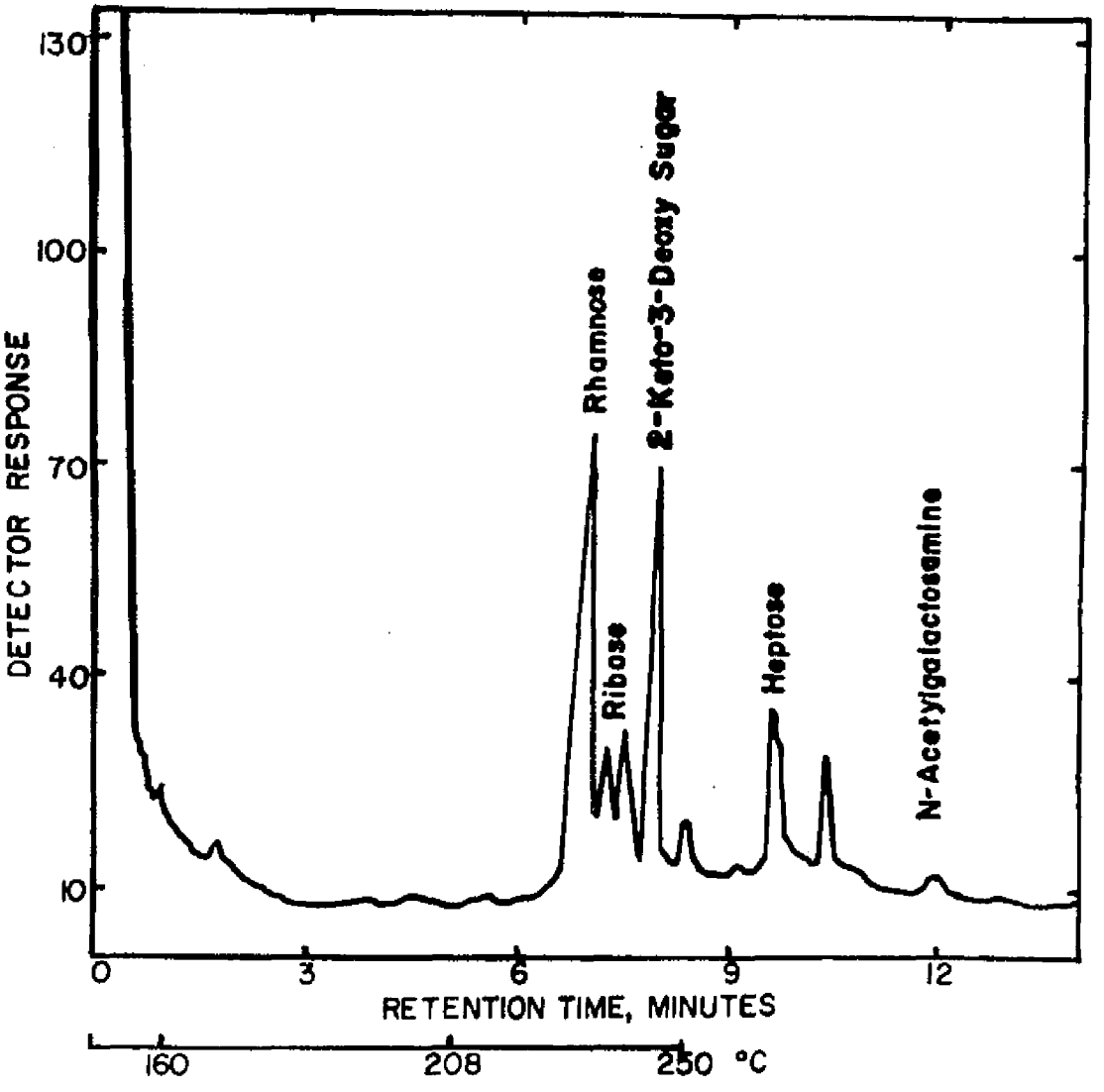

DETECTOR RESPONSE
130
100
70
40
10
Rhamnose
Ribose
Xylose
Galactosamine
Glucosamine or Galactose
0
3
6
9
12
RETENTION TIME, MINUTES
160
200
250 °C

Fig. 39. Analysis of LPS isolated from intact cysts of Azotobacter vinelandii by gas chromatography.


130
100
70
40
10
DETECTOR RESPONSE
Rhamnose
Ribose
2-Keto-3-Deoxy Sugar
Heptose
N-Acetylgalactosamine
0
3
6
9
12
RETENTION TIME, MINUTES
160
208
250 °C

assigned on the basis of relative retention times reported by other investigators (Weckesser, et al., 1972, and Hammerling, et al., 1971).

The lipid A component (fraction II) contained rhamnose, galactosamine, and N-acetylgalactosamine (Figure 40). Ethanolamine, 2-keto-3-deoxy sugar, and heptose peaks were assigned as indicated in the results obtained from LPS analysis. The additional peak at a retention time of 3.1 remained unidentified.

Preliminary gas chromatographic analysis of the methyl-ester fraction of lipid A (fraction I) indicates that both a C-12 and a C-14 fatty acid derivative are present.

The quantitative analysis of the saccharides in the various fractions by gas chromatography is presented in Table 9. Heptose, 2-keto-3-deoxy sugars, and ethanolamine are included in these results although their absolute identity is uncertain.

X-ray probe microanalysis

The action of calcium to bind to LPS polyanions suggested that cells and cysts of Azotobacter should contain differing amounts of calcium. Cells and cysts were washed free of culture medium and examined for calcium content by X-ray probe microanalysis. The results of the vegetative cell analysis is presented in Figure 41. The high magnesium, aluminum, manganese and iron peaks are present as components of the stub which served as the specimen holder. The two cell specific elements are potassium and calcium, both identified by their K_{α} peaks. When the calcium content of cysts was examined, the amount detected was considerably greater than that found in vegetative cells (Figure 42). Both the calcium K_{α} and K_{β} peaks were visible. Estimates of the

Fig. 40. Analysis of the lipid A component of the LPS from Azotobacter vinelandii by gas chromatography.

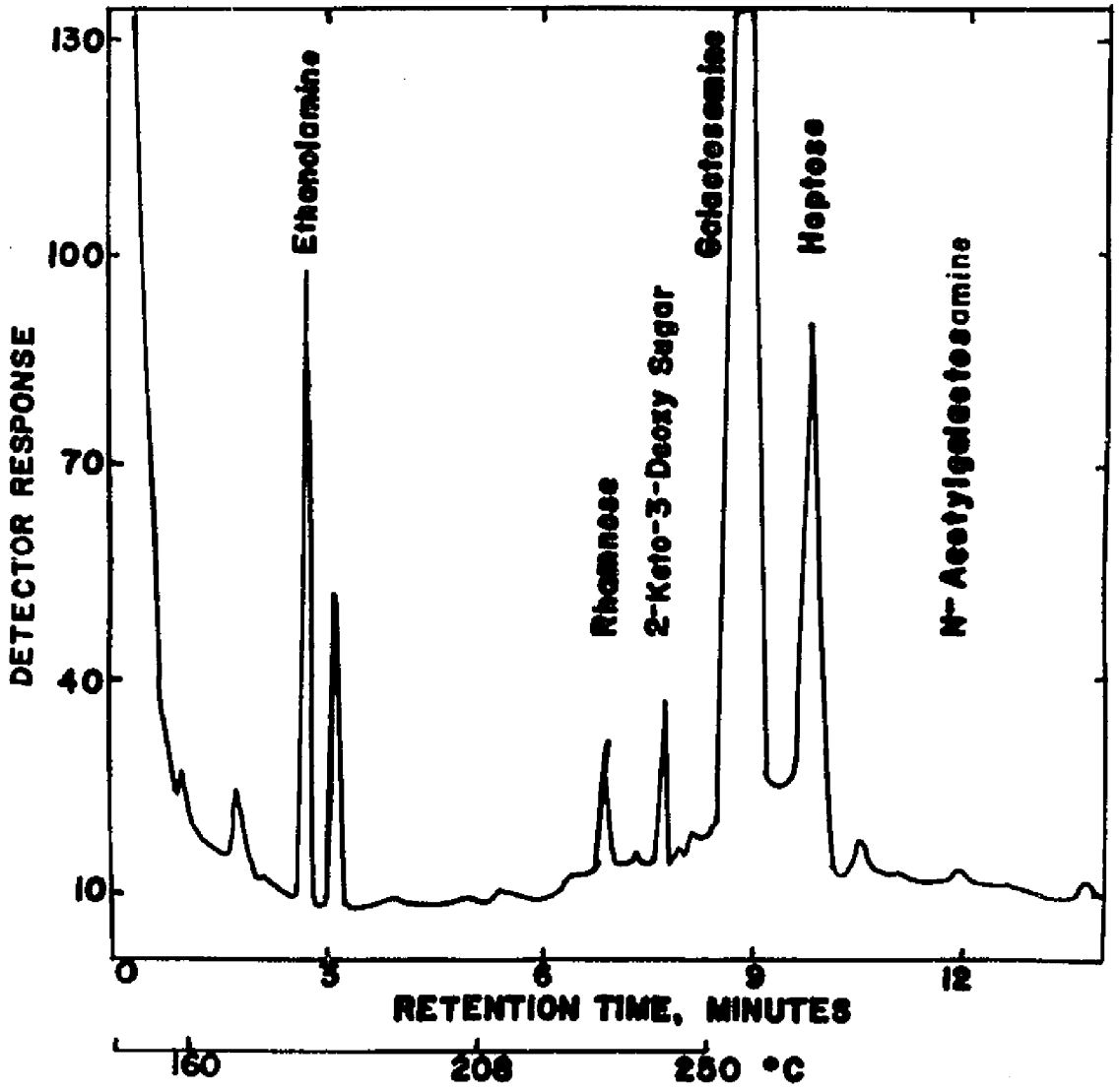

130
100
70
40
10
DETECTOR RESPONSE
Ethanolamine
Rhamnose
2-Keto-3-Deoxy Sugar
Galactosamine
Heptose
N-Acetylgalactosamine
0
3
6
9
12
RETENTION TIME, MINUTES
160
200
250 °C

Table 9. Gas chromatographic analysis of saccharides in hydrolysates of cyst coat fractions.

Component	Percentage				
	Exine	Intine	LPS	Lipid A	EDTA-LPS
Ribose	2.8	3.4	16.1		1.0
Xylose		5.3			1.4
Rhamnose	2.1	5.2	23.9	4.4	1.9
Glucose	10.1				
Galactose		33.6			6.3
Glucosamine	2.3				
Galactosamine		7.6		25.5	8.8
N-acetylgalactosamine	1.1	1.1	1.7	1.3	
Heptose			36.7	18.9	
Ethanolamine				+	
2-keto-3-deoxy sugar			+	+	

Fig. 41. X-ray probe microanalysis of calcium in vegetative cells of Azotobacter vinelandii.

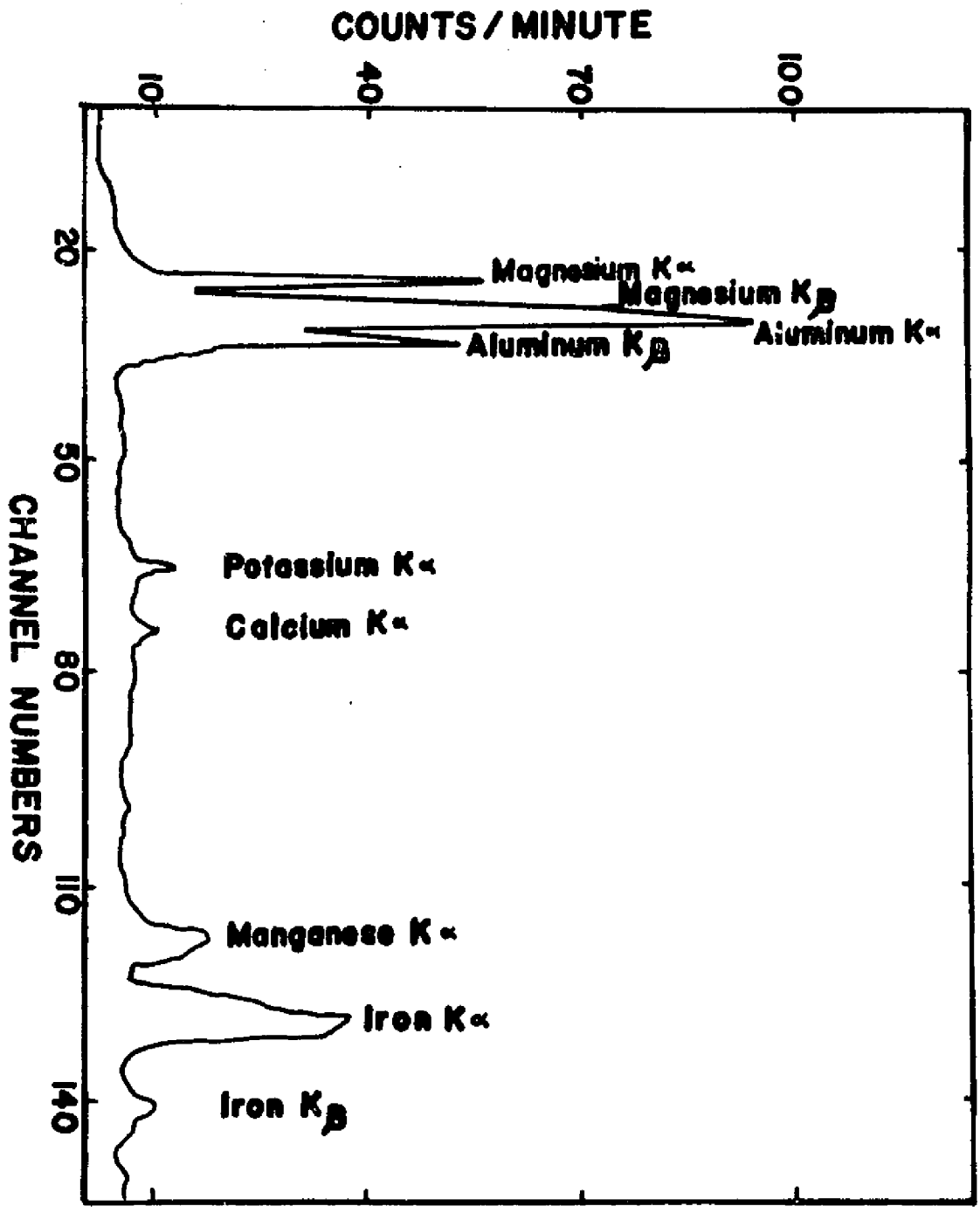
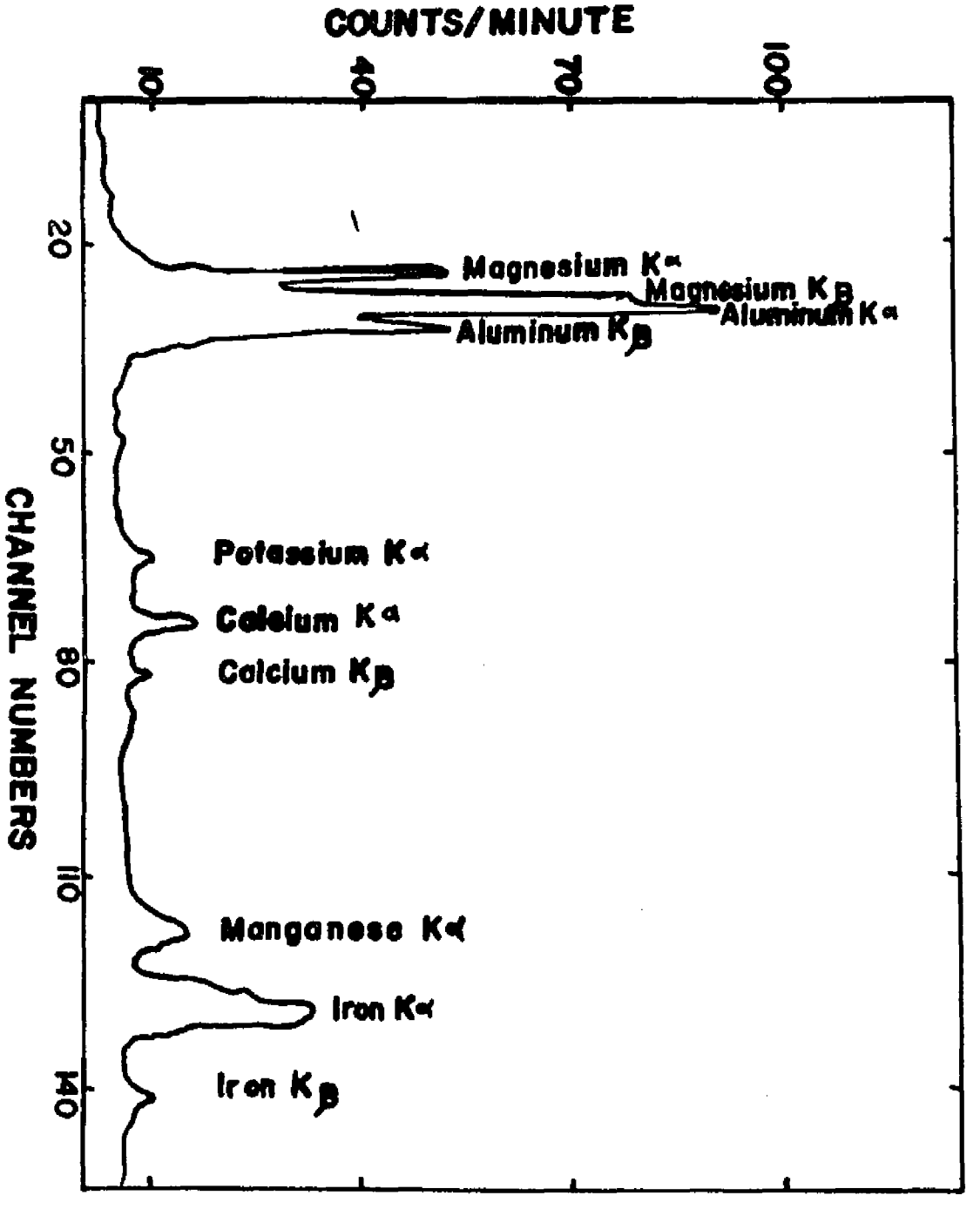

COUNTS / MINUTE
10
40
70
100
CHANNEL NUMBERS
20
50
80
110
140
Magnesium Kα
Magnesium Kβ
Aluminum Kα
Aluminum Kβ
Potassium Kα
Calcium Kα
Manganese Kα
Iron Kα
Iron Kβ

Fig. 42. X-ray probe microanalysis of calcium in cysts of Azotobacter vinelandii.


COUNTS/MINUTE
20
50
80
110
140
CHANNEL NUMBERS
Magnesium Kα
Magnesium Kβ
Aluminum Kα
Aluminum Kβ
Potassium Kα
Calcium Kα
Calcium Kβ
Manganese Kα
Iron Kα
Iron Kβ

peak areas indicate that cysts contain twice as much calcium as vegetative cells.

DISCUSSION

Cysts of Azotobacter vinelandii have been produced in fermentors containing Burk's nitrogen-free medium by the addition of 0.6% powdered $CaCO_3$ with 1% glucose as the source of carbon. When $CaCO_3$ was omitted from the medium, the pH rapidly fell to a level which did prevented proper encystment. The optimal pH for encystment appears to be 7.8, the final pH established in the presence of sufficient $CaCO_3$. Since $CaCO_3$ is extremely insoluble, its presence as a buffer in no way adversely affects the normal growth of the cells. Periodic adjustment of pH to 7.8 by alternate means failed to increase the efficiency of encystment, indicating that $CaCO_3$ serves an important function in production of cysts in addition to acid neutralization. The viscosity of nonencysting cultures became extremely high as a result of the excretion of acid polysaccharides into the culture medium. When sufficient calcium was present, these acid polysaccharides were not found in the medium.

The present study has demonstrated the incomplete formation of exine in BHB-supplemented cultures. Apparently the component necessary for final polymerization of this "fragile" exine is dependent upon the presence of sufficient calcium. Couperwhite and McCallum (1974) have shown that the composition of alginate of Azotobacter may be shifted from a predominance of guluronic acid by deletion or addition of calcium. Thus, calcium could serve a regulatory function by triggering compositional shifts of the alginate to polymers which

are incorporated into cyst coat. The absence of an appropriate level of calcium results in the so-called "abortive" encystment in which polymers characteristic of vegetative cells predominate. This condition occurs when cells were produced in the BHB-supplemented medium. Further studies concerning these cultures should be undertaken.

The presence of LPS in culture supernatants from both encysting and nonencysting cultures has been demonstrated. The disappearance of LPS from the culture medium during encystment is thought to result from coordination of this material into structural units of the cyst coat. Calcium ions have been shown to bind to purified LPS resulting in the ultrastructure observed in negative stains by electron microscopy. Another mechanism to account for the disappearance of LPS from the culture medium is possible. The presence of sufficient calcium could result in formation of LPS aggregates of sufficient size to be sedimented with the cell pellet upon centrifugation. This effect could explain the difference noted in residual LPS concentration between BHB-replacement cultures and cultures produced with free $CaCO_3$ in the medium. The presence and subsequent disappearance of LPS from BHB-replacement cultures indicates that removal of LPS from the culture medium is a necessary event in formation of normal cyst coat components.

A study of the sequential events in the morphogenesis of cells encysting in batch cultures utilizing 1% glucose as a source of carbon has demonstrated conclusively that cysts were produced by this method. This report contradicts the work of Lin and Sadoff (1968) who state that glucose is an inhibitor of encystment in Azotobacter.

Morphogenesis of glucose-grown cysts paralleled the development of cysts produced by the BHB-replacement method (Hitchins and Sadoff, 1970). The production of vesicles by encysting cells examined in thin sections was confirmed by scanning electron microscopic observations of critical point dried specimens. This technique of specimen preparation virtually eliminates production of artifacts resulting from surface tension during drying which so plagued Cagle, et al. (1973).

SEM observations provided the first visible evidence concerning the possible fate of flagella during encystment. Motile rod-shaped cells round up at initiation of encystment. Motility continues for the first few hours after encystment has been initiated. Phase microscopic observations indicate the spherical precysts tumble and spin, thereby wrapping the flagella around the cell. After flagellar movement has ceased, successive accumulations of capsule and cyst coat materials as observed by both TEM and SEM procedures, encase the flagella.

The double-fixation technique utilized for preparation of specimens for thin sectioning significantly reduced the introduction of artifacts frequently encountered with single-step fixation procedures. The structural integrity which was apparent enabled elucidation of the differentiation of cyst coat into alternating structures previously unreported. Ruthenium red stained the acid polysaccharide of the cyst coat and the capsule enabling resolution of the extracellular polysaccharides of cysts into the differentiated structures heretofore undescribed.

TEM observations provided information relating to the location of materials extracted from cysts by treatments with EDTA and phenol. Both of these extraction procedures removed a large portion of the area of the cyst coat between the exine and the central body. Leive (1965) reported that EDTA removes 50% of the LPS from the cell wall of gram negative organisms. The present study has demonstrated that EDTA also removes the intine structures of cyst coats of Azotobacter.

The intine component of cysts was isolated by two extraction methods utilizing EDTA. The method of Lin and Sadoff (1969a) resulted in complete solubilization of intine and intine vesicles. The material released could be precipitated from the EDTA solution with ethanol. This intine fraction is apparently identical to the EDTA-LPS fraction isolated from cysts by the method of Leive (1965). Both of these fractions possess the same chemical components when analyzed by gas chromatography (Table 9). The effect of EDTA on cyst coats and the similarity of the chemical composition suggests that intine is composed of LPS in addition to polysaccharides of capsular origin.

The action of phenol is more specific, removing only LPS from the cyst coat. The integrity retained by the cyst coat after treatment with phenol indicates that the polysaccharide component is unaffected. Only the vesicular and trilaminar components were removed by phenol. The L-layer of the cell wall of the central body has been removed by phenol treatment; however, numerous trilaminar structures are present between the central body and the cyst coat. Apparently these structures were unable to diffuse through the cyst coat. The low yield of LPS from intact cysts treated with phenol, compared to reported yields

from vegetative cells of Escherichia coli (Leive, et al., 1968), indicates that most of the LPS isolated, originated in the cyst coat. When the cyst coat was ruptured with EDTA prior to phenol treatment, the yield of LPS was noticeably higher.

Cysts produced in batch cultures possessed the sensitivity to EDTA characteristic of cysts produced on the solid medium with n-butanol as the source of carbon. Treatment with EDTA was utilized to rupture the cysts prior to separation of the exine and intine components for comparative chemical analysis. Attempts to isolate exine from cysts produced by other culture methods failed. Since the exine materials of cysts produced in the absence of sufficient calcium were poorly aggregated, the EDTA had little lytic effect, resulting in only partial rupture and incomplete release of the central body. The resulting components were poorly separated in linear sucrose gradients. If methods to increase the yield of exine produced in BHB-supplemented cultures could be developed, the analysis of this structure might provide information about the components and polymerization process necessary for complete formation of cyst coat.

The role of calcium to coordinate LPS into a sedimentable aggregate is consistent with the finding that LPS possesses ultrastructure in the presence of sufficient calcium. The purified LPS from Azotobacter formed trilaminar ribbons and rings when examined in negatively stained preparations by electron microscopy. The degree of aggregation of these structures was dependent upon the concentration of calcium or magnesium. When these divalent cations were absent, no ultrastructure was evident. The structures formed are typical of those seen in LPS derived from other organisms (Jackson and Zey, 1973).

The vesicular components observed in negative stains of EDTA-extracted intine material exhibited a complex structure indicating the presence of multiple components. Attempts to isolate intine vesicles as a particulate fraction by high speed centrifugation failed. A possible explanation for the presence of these vesicles in TEM preparations is based on the ability of the intine fraction to undergo spontaneous assembly to a stable structure in vitro. The complexity of these vesicles indicates that components in addition to LPS are present. This is supported by the findings of sugars from polysaccharides when intine was analysed by chromatographic procedures The capability of these macromolecules to form stable structures spontaneously or in the presence of divalent cations provides additional evidence for the role of calcium in encystment.

Partial identification of components in the cyst coat fractions has resulted in a better understanding of the origin and structure of cyst coats. Slime isolated from cultural supernatants of vegetative cell cultures contained glucose and a major component suspected to be a hexuronic acid. This latter component was present in greatly reduced amounts in the capsule of cells. The primary component present in slime from cyst cultures was glucose. Phase microscopic observations indicated the capsule of cysts remained virtually intact after several extractions with 10% NaCl adjusted to pH 9 with NaOH. Apparently the cyst capsule is of different composition from that of vegetative cells or it would be readily removed by the extraction procedure. This information supports the contention that calcium alters the composition of slime from Azotobacter. This altered slime could represent a primary component of the intine and exine. Further

studies are necessary to identify positively the components in cyst capsule so that they might be compared with the composition of vegetative slime and capsule.

Exine contained a suspected hexuronic acid component characteristic of the capsular polysaccharide. Apparently calcium effects a coordination of the exterior capsule polysaccharide to form exine. The resistance of exine to EDTA and its electron-dense appearance in thin sections indicate that calcium is tightly bound to the polysaccharide at the periphery of the cell. Further evidence for the specific localization of calcium could be obtained by analysis of spot maps and line scans produced during X-ray probe microanalysis.

Intine composition differed considerably from exine, including saccharides found in the capsule in addition to LPS-specific components. These LPS-specific components of cysts were found to be the same as those reported for vegetative cells by Olins and Warner (1967). The LPS of the present study contained rhamnose, ribose, heptose, a 2-keto-3-deoxy sugar, and galactosamine in addition to an unidentified component.

Claus (1965) reported the presence of 2-keto-3-deoxygalactonic acid as a capsular component of Azotobacter. The present study indicates that this component was probably the result of excretion of LPS into the medium under nonencysting conditions. Absolute identification of the heptose, hexuronic acid, and the 2-keto-3-deoxy component should be accomplished using authentic standards so that structural studies could be undertaken. Generally the compositions of cyst coat fractions determined by paper or gas chromatography were

identical. Where discrepancies were noted, the gas chromatography analysis is reported because of its greater sensitivity and specificity.

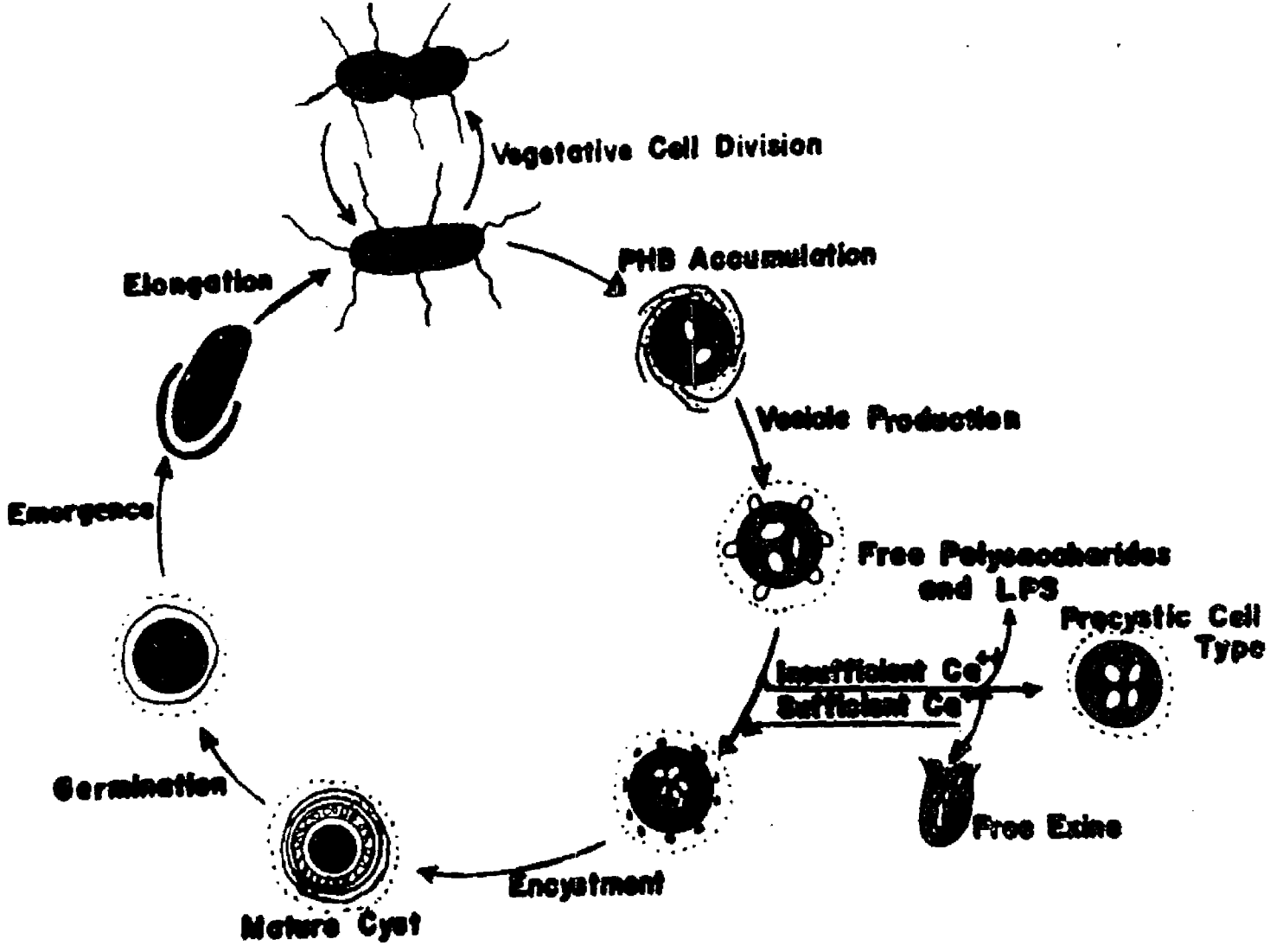
Lin and Sadoff (1969b) reported the amino composition for exine. They contend that the amino acids present represent the contribution of peptidoglycan. The present study fails to confirm this finding. Results presented here suggest that the minor protein component present probably results from flagella entrapped in the cyst coats during morphogenesis. Since the amino acid composition of flagella from Azotobacter remains unknown, no comparative studies were undertaken.

Attempts to determine the relative amounts of calcium present in vegetative cells and cysts of Azotobacter by X-ray probe micro-analysis indicated cysts contain more than twice the amount of calcium found in vegetative cells.

The summation of these data provides information necessitating a modification of the diagrammatic representation of the cyst developmental cycle presented by Stevenson (1967). This modified diagram is presented in Figure 43. Motile vegetative cells round up and accumulate PHB. Flagella become wrapped around the precyst, which becomes nonmotile. Vesicles originate from the L-layer of the cell wall and accumulate in the capsule adjacent to the cell. If sufficient calcium is present, the LPS and the altered slime are coordinated to form the differentiated layers of the cyst coat. Encystment is complete by the sixth day of growth.

When insufficient calcium is present, the LPS and slime accumulate in the medium and act to increase viscosity and lower pH

Fig. 43. Diagrammatic representation of encystment and germination of Azotobacter vinelandii.


Vegetative Cell Division
PHB Accumulation
Vesicle Production
Free Polysaccharides and LPS
Precystic Cell Type
Insufficient Ca++
Sufficient Ca++
Free Exine
Encystment
Mature Cyst
Germination
Emergence
Elongation

of cultures to nonencysting levels. The precystic cell types are occasionally surrounded by a loosely-defined exine-like structure and numerous exine-like structures appear throughout the culture medium.

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VITA

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EXAMINATION AND THESIS REPORT

Candidate: Nelson Peffley Moyer III

Major Field: Microbiology

Title of Thesis: Studies on the Encystment of Azotobacter vinelandii

Approved:

W D Sockloff
Major Professor and Chairman

James G. Traynham
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Date of Examination:

July 19, 1974